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(54) Title: PRODUCTION OF PROTEINS IN PLANT SEEDS (57) Abstract Methods for producing proteins in plant seeds are disclosed. Expression of the protein is driven by a seed-specific promoter and the protein is preferably expressed as a fusion polypeptide that includes a signal peptide that causes the protein to accumulate in a subcellular compartment to protect the protein. Also disclosed are improved methods for transforming plants, including cereals such as barley, corn or wheat.		

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PRODUCTION OF PROTEINS IN PLANT SEEDS

BACKGROUND OF THE INVENTION

5 Expression of Heterologous Proteins in Plant Seeds

The expression of heterologous proteins in plant seeds offers the possibility of, for example, producing large quantities of easily harvested polypeptides, and of expressing proteins that improve their grain quality. Discussions of this concept can be found in U.S. Patent No. 5,714,474 ("Production of enzymes in seeds and their uses").

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Hordein Storage Proteins

Barley seed storage proteins account for about 8 to 15% of the dry weight of the mature barley grain. The major seed storage proteins in barley are alcohol-soluble prolamines, termed hordeins, which are classified into two major groups, B and C, and two minor groups, D and γ (Shewry 1993). Depending on nitrogen levels, these four groups account for about 35 to 55% of total barley seed protein. The B- and C-hordeins account for about 70 to 80% and 10 to 20%, respectively, of the total hordein fraction, with small amounts of D- (2-4%) and γ -hordeins (not precisely determined). The B-, D- and γ -hordeins are sulfur-rich prolamines while the C hordeins are sulfur-poor prolamines (Bright and Shewry 1983). The hordeins are coordinately synthesized in the developing starchy endosperm tissue (Giese et al. 1983; Sørensen et al. 1989). They are cotranslationally transported into the lumen of the rough endoplasmic reticulum, with simultaneous cleavage of the signal peptide, and are ultimately deposited into protein bodies (Cameron-Mills 1980; Cameron-Mills and von Wettstein 1980; Cameron-Mills and Madrid 1989).

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Genetic analyses show that all hordeins are encoded by structural genes on chromosome 5 (1H) of barley; the *Hor1*, *Hor2*, *Hor3* and *Hor5* loci on chromosome 5 encode the C-, B-, D-, and γ -hordein polypeptides, respectively (Jensen et al., 1980; Shewry et al. 1980; Blake et al. 1982; Shewry et al. 1983; Shewry and Parmar 1987). The genes for B-, C- and D-hordeins have been isolated and characterized (Brandt et al. 1985; Forde et al. 1985; Rasmussen and Brandt 1986; Sørensen et al. 1996). The B- and C-hordeins are encoded by multigene families comprising 10 to 20 members while D-hordein is encoded by a single gene (Brandt et al. 1985; Rasmussen and Brandt 1986; Sørensen et al. 1996). The regulation and expression of these hordein promoters have been studied by transient expression assays (Entwistle et al. 1991; Müller and Knudsen 1993; Sørensen et al. 1996) in barley endosperm. As determined by these assays using promoter-*uidA* fusions, the D-hordein promoter is 3- to 5-fold more active than the B- or C-hordein promoters tested (Sørensen et al. 1996). The B-hordein promoter has also been studied using stable tobacco transformation with promoter-*cat* fusions (Marris et al. 1988).

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Although the genes for B-, C- and D-hordeins have been isolated and characterized, their regulation and expression have been studied only in transient expression assays in barley and in

stably transformed tobacco (Brandt et al., 1985; Forde et al., 1985; Marris et al., 1988 Sørensen et al., 1996).

5 In barley, wheat and maize, the major highly insoluble prolamin storage proteins are synthesized on polysomes closely associated with the endoplasmic reticulum (ER). (See Seeds: Physiology of Development and Germination, 2nd ed., eds. Bewley and Black, Plenum Press, New York, 1994). Newly synthesized proteins pass through the ER membrane into the lumen, where they aggregate into small particles, which eventually form larger aggregates and protein bodies (which can be observed in electron micrographs).

10 In wheat, two different types of protein bodies accumulate independently within the developing endosperm: low density bodies that develop earlier and high density bodies that develop later and are derived from the ER. The high density proteins are formed when aggregation of proteins inside the lumen of the ER puts a strain on the membrane and cause it to rupture. The membrane may reform free of the protein aggregate, after an interval in which the protein body itself is not bounded by a membrane. In other cereals besides wheat and barley, such as millet, rice, maize and sorghum, the protein bodies remain as distinct membrane-bound entities even in mature seed.

SUMMARY OF THE INVENTION

The present invention provides recombinant nucleic acid molecules that employ a seed maturation specific promoter linked to a polypeptide, and particularly to a construct that also includes
20 a signal sequence that targets a linked polypeptide to an intracellular body, such as a protein body. Such constructs may be represented as P – X or P – SS – X wherein P is a seed maturation-specific promoter, SS is a signal sequence, such as a sequence that targets a linked polypeptide to an intracellular body, and X is a polypeptide to be expressed in seed or a plant embryo. In particular
25 embodiments, X is a non-storage protein, which is targeted to a protein storage body. Seeds expressing polypeptides of interest may be harvested at preselected times that have been found to provide greatest expression or stability of the polypeptide.

In particular embodiments, the invention provides recombinant nucleic acid molecules that employ hordein promoters, such as hordein B₁ and D promoters, to direct expression of polypeptides
30 in seeds of transgenic plants, including monocot plants. In particular, the invention provides nucleic acid molecules in which a hordein signal sequence is operably linked to a nucleic acid sequence encoding the polypeptide of interest. It is shown that inclusion of a hordein signal sequence can significantly increase the levels of the expressed polypeptide in the plant seed. A wide range of polypeptides may be expressed in plant seeds in this manner, including pharmaceuticals such as
35 insulin, interferons, erythropoietin and interleukins, and nutritional supplements.

The nucleic acid molecules provided by this aspect of the invention may be represented as

Ph - hSS - X

wherein Ph is a hordein promoter, hSS is a hordein signal sequence, and X is a nucleic acid molecule encoding a polypeptide (particularly a polypeptide that is not a seed storage protein), and where Ph, hSS and X are operably linked. Nucleic acid molecules lacking the hordein signal sequence may be represented as:

Ph - X.

The invention provides transgenic plants comprising these nucleic acid molecules, as well as seeds of these transgenic plants, which are useful as a source of the expressed polypeptide, or may improve the quality of the grain.

In particular embodiments of the invention, the transgenic plants provided are stably transformed monocotyledonous plant, for example cereal plants, such as barley or wheat. In particular embodiments, the invention provides stably transformed barley plants from genotypes including: Harrington, Morex, Crystal, Stander, Moravian III, Galena, Salome, Steptoe, Klages and Baronesse. The invention also provides stably transformed wheat plants from genotypes including: Anza, Karl, Bobwhite and Yecora Rojo. Most of these genotypes are not amenable to conventional transformation procedures. Accordingly, in order to enable production of stably transformed plants belonging to these genotypes, the invention also provides a transformation method that, in conjunction with the nucleic acid molecules provided, may be employed to produce the stably transformed plants. This transformation method is based upon the production of green regenerable tissues from zygotic plant embryos, and may be used to transform any monocot species, including maize, wheat, barley, rice, oat, rye, millet, sorghum, tricalate, turfgrass and forage grasses.

The transformation method comprises

(a) placing an immature zygotic embryo of the selected monocot plant on plant growth medium comprising maltose as a sugar source, an auxin at a concentration of about 0.1 mg/L to about 5 mg/L, a cytokinin at a concentration of 0 mg/L to about 5 mg/L and copper at a concentration of about 0.1 μ M to about 50 μ M, and incubating in dim light conditions so as to form green regenerative tissue;

(b) introducing a nucleic acid molecule into the tissue by to produce transformed tissue; the nucleic acid may be either

P - hSS - X,

P - X,

Ph - hSS - X, or

Ph - X

wherein P is a seed maturation-specific promoter, SS is a signal sequence that targets a polypeptide to an intracellular body (such as a protein body or vacuole), Ph is a hordein promoter (a particular seed maturation specific promoter), hSS is a hordein signal sequence, and X is a nucleic acid molecule encoding the selected polypeptide (which may be other than a seed storage protein), and

where Ph, hSS and X (or Ph and X) are operably linked;

(c) incubating the transformed tissue on the plant growth medium such that green structures are observed on the transformed material;

(d) regenerating at least one transformed plant from the green structures; and

(f) growing the transformed plant to produce seed.

The invention also provides seeds of stably transformed plants that express the selected polypeptide in their seed. Another aspect of the invention is a method of expressing a polypeptide in seed of a monocotyledenous plant, comprising providing a monocotyledenous plant that is stably transformed with a nucleic acid molecule of structure Ph - hSS - X, or Ph - X, and growing the plants under conditions effective to produce seed and express the polypeptide in the seed. The polypeptide can be used to improve the quality of the grain, or it can be extracted from the seed at a time of maximum expression or stability to be used for other purposes.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A is a schematic diagram of a four-primer strategy for producing chimeric products using the polymerase chain reaction (PCR).

Fig. 1B is a schematic diagram of a three-primer strategy for producing chimeric products using the polymerase chain reaction (PCR).

Fig. 1C is a map of a construct that includes (from 5' to 3'), the B₁-hordein promoter, the B₁-hordein signal sequence, the *uidA* gene, and the *nos* 3' terminator.

Figs. 2A and B show alignments of constructs comprising the B₁-hordein promoter, the *uidA* gene, and the *nos* 3' terminator, with (2A) or without (2B) the B₁-hordein signal sequence.

Fig. 3 shows the nucleic acid sequence of the B₁-hordein promoter and the 57 base pair B₁-hordein signal sequence (underlined).

Fig. 4 shows the nucleic acid sequence of the D-hordein promoter and the 63 base pair D-hordein signal sequence (underlined).

Fig. 5 is a bar graph showing GUS activity in mature barley seeds expressing constructs comprising the B₁-hordein promoter, the *uidA* gene, and the *nos* 3' terminator either with (+SS) or without (-SS) the B₁-hordein signal sequence.

Fig. 6 is a bar graph showing GUS activity in immature barley seeds expressing constructs comprising the B₁-hordein promoter, the *uidA* gene, and the *nos* 3' terminator either with (+SS) or without (-SS) the B₁-hordein signal sequence.

Fig. 7 is an electron photomicrograph in which an immunosignal is specific for protein bodies in GUS-expressing immature endosperm of a line that was transformed with the B₁-hordein-*uidA* DNA construct containing the 19 amino acid N-terminal signal peptide sequence.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids. Only one strand of each nucleic acid sequence is shown, but it is understood that the complementary strand is included by any reference to the displayed strand.

Seq. I.D. No. 1 shows the nucleic acid sequence of the barley B₁ hordein promoter and signal sequence.

Seq. I.D. No. 2 shows the amino acid sequence of the barley B₁ hordein signal sequence.

Seq. I.D. No. 3 shows the nucleic acid sequence of the barley D hordein promoter and signal sequence.

Seq. I.D. No. 4 shows the amino acid sequence of the barley D hordein signal sequence.

Seq. I.D. Nos. 5- 16 show PCR primers used to amplify nucleic acid molecules as described herein.

DETAILED DESCRIPTION OF THE INVENTION

I. Abbreviations and Definitions

A. Abbreviations

HMW: high molecular weight
 CAT: chloramphenicol acetyl transferase
 GUS: β -glucuronidase
uidA: β -glucuronidase gene
 PCR: polymerase chain reaction
 PEG: polyethylene glycol
 MS medium: Murashige and Skoog medium
 CIM: callus induction medium
 IIM: intermediate-incubation medium
 RM: regeneration medium
 2,4-D: 2,4-dichlorophenoxyacetic acid
 BAP: 6-benzylaminopurine
 2iP: N⁶-(2-isopentyl)adenine
 GFP: green fluorescent protein
 CaMV: cauliflower mosaic virus
 rbcS: RUBISCO (D-ribulose-1,5-bisphosphate carboxylase/oxygenase) small subunit

B. Definitions

Unless otherwise noted, technical terms are used according to conventional usage.

Definitions of common terms in molecular biology may be found in Lewin, *Genes V* published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew et al (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of the invention, the following definitions of terms are provided:

Promoter: A nucleic acid sequence that directs transcription of a protein. This includes not only molecules having the prototypical sequences, but also promoters from gene homologs. Also included are molecules that differ from the disclosed prototypical molecules by minor variations. Such variant sequences may be produced by manipulating the nucleotide sequence of a promoter using standard procedures.

Hordein promoter: A nucleic acid sequence that directs transcription of a hordein protein in seeds of a plant. While any hordein promoter may be employed for this invention, the specific examples provided describe the use of the promoter sequences from the B₁ and D hordein genes of barley. The nucleic acid sequences of the prototypical barley B₁ and D hordein genes are shown in Seq. I.D. Nos. 1 and 3, respectively as well as in Figs. 3 and 5, respectively. The promoter region excludes those nucleotides that encode the signal sequence (the underlined sequences shown in Figs. 3 and 4). One of skill in the art will appreciate that the length of the promoter region may also be greater or less than the sequences depicted. For example, additional 5' sequence from the hordein gene upstream region may be added to the promoter sequence, or bases may be removed from the depicted sequences. However, any hordein promoter sequence must be able to direct transcription of an operably linked sequence in plant seed. The ability of a barley hordein promoter to direct transcription of a protein in a plant seed may readily be assessed by operably linking the promoter sequence to an open reading frame (ORF) (preferably encoding a readily detectable protein) such as the *GUS* open reading frame, introducing the resulting construct into plants and then assessing expression of the protein in seeds of the plant, as described in detail below. A hordein promoter will typically confer seed-specific expression, meaning that expression of the protein encoded by the operably linked ORF will generally be at least twice as high (assessed on an activity basis) in seeds of the stably transfected plant compared to other tissues such as leaves. More usually, the hordein promoter will produce expression in seeds that is at least 5 times higher than expression in other tissues of the plant. In many cases, the expression of the protein in the seed will be endosperm-specific.

Functional homologs of the barley hordein promoters disclosed herein may be obtained

from other plant species, such as from other monocots, including wheat, rice and corn. Specific examples of such homologs may have specified levels of sequence identity with the prototype hordein promoters (e.g., at least 60% sequence identity). the functional homologs retain hordein promoter function, i.e., retain the ability to confer seed specific expression on operably linked ORFs when introduced into plants. Accordingly, where reference is made herein to a hordein promoter, it will be understood that such reference includes not only molecules having the sequences of the prototypical sequences disclosed herein (or variations on these sequences), but also promoters from hordein gene homologs. Also included within the scope of such terms are molecules that differ from the disclosed prototypical molecules by minor variations. Such variant sequences may be produced by manipulating the nucleotide sequence of hordein promoter using standard procedures such as site-directed mutagenesis or the polymerase chain reaction.

Hordein signal sequence (SS): The inventors have discovered that the inclusion of a hordein signal sequence in conjunction with a hordein promoter provides enhanced expression of certain heterologous proteins in seeds. In particular, the expression of a protein in immature seeds is greatly enhanced when the ORF encoding the protein is operably linked to both a hordein promoter and a hordein signal sequence, compared with an equivalent construct in which the hordein signal sequence is absent. While not wishing to be bound by speculation, it is proposed that the hordein signal sequence directs expression of a protein encoded by an operably linked ORF to a protected subcellular location, such as a vacuole or protein body. It is further proposed that proteins directed to such vacuoles are protected from proteolysis during certain stages of seed maturation.

The hordein signal sequence typically comprises about the first 15-25 amino acids of the hordein gene open reading frame, more usually about 18-21 amino acids. The nucleotide and amino acid sequences of the hordein signal sequences of the prototypical barley B, and D hordein genes are shown in Seq. I.D. Nos. 1-4. One of skill in the art will appreciate that while these particular signal sequences are utilized in the examples described below, the invention is not limited to these specific sequences. For example, homologous sequences may be used as effectively, as may sequences that differ in exact nucleotide or amino acid sequences, provided that such sequences result in enhanced levels of the encoded protein in immature seeds. Typically, "enhanced expression" will be expression that is about twice that observed with an equivalent construct lacking the signal sequence. Accordingly, the term "hordein signal sequence" includes not only the particular sequences shown herein, but also homologs and variants of these sequences.

Sequence identity: The similarity between two nucleic acid sequences, or two amino acid sequences is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more similar the two sequences are. Homologs of the prototype hordein promoters and hordein signal sequences will possess a relatively high degree of sequence identity when aligned using standard methods.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith and Waterman (1981); Needleman and Wunsch (1970); Pearson and Lipman (1988); Higgins and Sharp (1988); Higgins and Sharp (1989); Corpet et al. (1988); Huang et al. (1992); and Pearson et al. (1994). Altschul et al. (1994) presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. It can be accessed at <http://www.ncbi.nlm.nih.gov/BLAST/>. A description of how to determine sequence identity using this program is available at http://www.ncbi.nlm.nih.gov/BLAST/blast_help.html.

Homologs of the disclosed prototype hordein signal sequences are typically characterized by possession of at least 60% sequence identity counted over the full length alignment with the amino acid sequence of the prototype using the NCBI Blast 2.0, gapped blastp set to default parameters. Proteins with even greater similarity to the reference sequences will show increasing percentage identities when assessed by this method, such as at least 70%, at least 75%, at least 80%, at least 90% or at least 95% sequence identity. Homologous hordein promoters include those from genes that encode proteins having equivalent levels of sequence identity with the B₁ and D hordein proteins (i.e., at least 60% and up to at least 95%).

Oligonucleotide: A linear polynucleotide sequence of up to about 100 nucleotide bases in length.

Vector: A nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector may include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication. A vector may also include one or more selectable marker genes and other genetic elements known in the art.

Transformed: A transformed cell is a cell into which has been introduced a nucleic acid molecule by molecular biology techniques. As used herein, the term transformation encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including transfection with viral vectors, transformation with plasmid vectors, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration, and includes transient as well as stable transformants.

Isolated: An "isolated" biological component (such as a nucleic acid or protein or organelle) has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extra-chromosomal DNA and RNA, proteins and organelles. Nucleic acids and proteins that have been "isolated" include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as

chemically synthesized nucleic acids.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

Recombinant: A recombinant nucleic acid is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques.

cDNA (complementary DNA): A piece of DNA lacking internal, non-coding segments (introns) and regulatory sequences that determine transcription. cDNA is synthesized in the laboratory by reverse transcription from messenger RNA extracted from cells.

ORF (open reading frame): A series of nucleotide triplets (codons) coding for amino acids without any termination codons. These sequences are usually translatable into a peptide.

Transgenic plant: As used herein, this term refers to a plant that contains recombinant genetic material not normally found in plants of this type and which has been introduced into the plant in question (or into progenitors of the plant) by human manipulation. Thus, a plant that is grown from a plant cell into which recombinant DNA is introduced by transformation is a transgenic plant, as are all offspring of that plant that contain the introduced transgene (whether produced sexually or asexually).

The term "transgenic plant" also encompasses parts of plants, including fruit, seeds and pollen.

The present invention is applicable to both dicotyledonous plants (e.g., tomato, potato, soybean, cotton, tobacco, etc.) and monocotyledonous plants, including, but not limited to, graminaceous monocots such as wheat (*Triticum* spp.), rice (*Oryza* spp.), barley (*Hordeum* spp.), oats (*Avena* spp.), rye (*Secale* spp.), corn (*Zea mays*), sorghum and millet (*Pennisetum* spp). For example, the present invention can be employed with barley genotypes including, but not limited to, Morex, Harrington, Crystal, Stander, Moravian III, Galena, Salome, Steptoe, Klages, Baronesse, and with wheat genotypes including, but not limited to Yecora Rojo, Bobwhite, Karl and Anza. In general, the invention is particularly useful in cereals.

Seed maturation: Seed maturation or grain development refers to the period starting with fertilization in which metabolizable food reserves (e.g., proteins, lipids, starch, etc.) are deposited in the developing seed, particularly in storage organs of the seed, including the endosperm, testa,

aleurone layer, embryo, and scutellar epithelium, resulting in enlargement and filling of the seed and ending with seed desiccation.

Inducible: Characterized by a promoter that is upregulated by the presence or absence of a small molecule; it includes both direct and indirect inducement.

5 **Seed maturation specific promoter:** A promoter induced during seed maturation, for example increased by 25% or more during seed maturation.

Signal/leader/target/transport sequence: An N-terminal or C-terminal polypeptide sequence which is effective to co-translationally or post-translationally localize the polypeptide or protein to which it is attached to a selected intracellular vacuole or other protein storage body, chloroplast, mitochondria, or endoplasmic reticulum, or extracellular space or seed region, such as the endosperm, following secretion from the cell. An example in barley is the hordein signal sequence, but other examples include signal sequences in maize (Bagga et al., *Plant Cell* 9:1683-1696, 1997 in which coexpression of the maize delta-zein and beta-zein genes results in stable accumulation of delta-zein in endoplasmic reticulum-derived bodies formed by beta-zein; Torrent et al., *Plant Molecular Biology* 34:139-149, 1997 in which lysine-rich modified gamma-zeins accumulate in protein bodies of transiently transformed maize endosperms); in rice (Wu et al., *Plant Journal* 14:673-683, 1998 in which the GCN4 motif is essential for endosperm-specific gene expression, and is activated by Opaque-2, in transgenic rice plants; Zheng et al., *Plant Physiology* 109:777-786, 1995 in which bean beta-phaseolin was expressed in transgenic rice endosperm, primarily in the vacuolar type-II protein bodies near the aleurone layer); in wheat (Grimwade et al., *Plant Molecular Biology* 30:1067-1073, 1996 in which expression patterns of genes coding for wheat gluten proteins were described); in tobacco with legumin (Conrad et al., *Journal of Plant Physiology* 152:708-711, 1998) which discloses large-scale production of pharmaceutical proteins in transgenic tobacco plants using two seed-specific *Vicia faba* promoters from the legumin B4 so that the product was retained in the endoplasmic reticulum); and soy engineered using the lectin gene (Takaiwa et al., *Plant Science* 111:39-49, 1995 in which soybean glycinin genes, transcriptionally fused to an endosperm-specific promoter of the rice storage protein glutelin gene, were introduced into the tobacco genome via *Agrobacterium*-mediated transformation, and were expressed specifically in the cotyledon and embryo of maturing soybean seed).

30 **Terminal processing or termination sequence:** A DNA sequence located 3' to the coding region which causes RNA polymerase to terminate transcription of a gene, and dissociate from the DNA. An example is the *nos* 3' terminator. A terminator may also occur during post-transcription processing of mRNA in vivo, as indicating where a precursor mRNA molecule should be cleaved to yield a mature mRNA species which will be translated. A segment containing a terminal-processing signal can be obtained by comparing cDNA encoding an endosperm-expressed product coding for the same product, thereby to identify the 3' terminus of the cDNA. By isolating a genomic DNA

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segment comprising 50 to 100 base-pairs on either side of the 3' terminus, one is assured of obtaining a segment with the terminal-processing signal.

Protein body: Intracellular structure containing proteins within a unit membrane structure. In some cases this structure is referred to as a vacuole.

Seed storage protein: An endogenous plant protein which is synthesized and accumulated during seed maturation, stored in the dry grain, and mobilized during maturation. Such proteins are often stored in a protein body in a plant seed. Examples of such storage proteins include arachin, avenin, cocosin, conarachin, concocoin, conglutin, conglycinin, convicine, crambin, cruciferin, cucurbitin, edestin, excelesin, gliadin, gluten, glytenin, glycinin, helianthin, hordein, kafirin, legumin, napin, oryzin, pennisetin, phaseolin, psophocarpin, secalin, vicilin, vicine and zein.

II. Seed-Specific Expression of Proteins Using Hordein Promoter/Hordein Signal Sequence Constructs

a. Constructs

The present invention provides recombinant constructs that are suitable for obtaining high level expression of a specified polypeptide in plant seeds. The constructs may be generally represented as Ph - hSS - X, wherein Ph is a hordein promoter, hSS is a hordein signal sequence, and X is a nucleic acid molecule encoding the specified polypeptide. Each of these three components is operably linked to the next, i.e., the hordein promoter is linked to the 5' end of the sequence encoding the hordein signal sequence, and the hordein signal sequence is operably linked to the X sequence. The construct will usually also contain 3' regulatory sequences, such as the Nos 3' region.

The characteristics of hordein promoters and signal sequences are described above. The B1 and D hordein genes are described in Brandt et al. (1985) and Sørensen et al. (1996). When the promoter is Ph, the polypeptide "X" may be any polypeptide except for a hordein polypeptide, and in particular embodiments is other than a seed storage protein, or even a seed specific protein. Polypeptides X that may be expressed in plant seeds as described herein, as part of a P-X, P-SS-X, Ph-X or Ph-SS-X construct include non seed specific proteins such as human therapeutic proteins (e.g., erythropoietin, tissue plasminogen activator, urokinase and prourokinase, growth hormones, cytokines, factor VIII, epoetin- α , granulocyte colony stimulating factor, antibodies, vaccines, etc.), or more plant specific proteins such as enzymes for starch biosynthesis (e.g., ADP glucosepyrophosphorylase, EC 2.7.7.27; starch synthase, EC 2.4.1.21; and branching enzyme, R,Q) and seed specific proteins, such as those conferring enhanced nutritional value on the seeds. Nucleic acids encoding such proteins are well known in the art. The coding region for such a protein may be modified such that it more closely conforms to the preferred codon usage bias for a particular host cell.

Other heterologous proteins encoded by the chimeric gene include polypeptides that form immunologically active epitopes, and enzymes that catalyze conversion of intracellular metabolites, with the consequent build-up of selected metabolites in the cells.

The expression cassette or chimeric genes in the transforming vector typically have a transcriptional termination region at the opposite end from the transcription initiation regulatory region. The transcriptional termination region may normally be associated with the transcriptional initiation region from a different gene. The transcriptional termination region may be selected, particularly for stability of the mRNA, to enhance expression. Illustrative transcriptional termination regions include the NOS terminator from *Agrobacterium* Ti plasmid and the rice α -amylase terminator.

Polyadenylation tails are also commonly added to the expression cassette to optimize high levels of transcription and proper transcription termination, respectively.

Standard molecular biology methods, such as the polymerase chain reaction may be employed to produce these constructs.

b. General Principles of Plant Transformation

Introduction of the Ph – hSS – X construct into plants is typically achieved using standard techniques. The basic approach is to clone the construct into a transformation vector which is then introduced into plant cells by one of a number of techniques (e.g., electroporation) and progeny plants containing the introduced construct are selected. Preferably all or part of the transformation vector will stably integrate into the genome of the plant cell. That part of the transformation vector which integrates into the plant cell and which contains the introduced Ph – hSS – X sequence (the introduced “transgene”) may be referred to as the recombinant expression cassette.

Selection of progeny plants containing the introduced transgene may be made based upon the detection of protein X expression or seeds, or upon enhanced resistance to a chemical agent (such as an antibiotic) as a result of the inclusion of a dominant selectable marker gene incorporated into the transformation vector.

Successful examples of the modification of plant characteristics by transformation with cloned nucleic acid sequences are replete in the technical and scientific literature. Selected examples, which serve to illustrate the knowledge in this field of technology include:

U.S. Patent No. 5,571,706 (“Plant Virus Resistance Gene and Methods”)

U.S. Patent No. 5,677,175 (“Plant Pathogen Induced Proteins”)

U.S. Patent No. 5,510,471 (“Chimeric Gene for the Transformation of Plants”)

U.S. Patent No. 5,750,386 (“Pathogen-Resistant Transgenic Plants”)

U.S. Patent No. 5,597,945 (“Plants Genetically Enhanced for Disease Resistance”)

U.S. Patent No. 5,589,615 (“Process for the Production of Transgenic Plants with Increased Nutritional Value Via the Expression of Modified 2S Storage Albumins”)

U.S. Patent No. 5,750,871 (“Transformation and Foreign Gene Expression in Brassica Species”)

U.S. Patent No. 5,268,526 (“Overexpression of Phytochrome in Transgenic Plants”)

U.S. Patent No. 5,780,708 ("Fertile Transgenic Corn Plants")

U.S. Patent No. 5,538,880 ("Method For Preparing Fertile Transgenic Corn Plants")

U.S. Patent No. 5,773,269 ("Fertile Transgenic Oat Plants")

U.S. Patent No. 5,736,369 ("Method For Producing Transgenic Cereal Plants")

U.S. Patent No. 5,610,042 ("Methods For Stable Transformation of Wheat").

These examples include descriptions of transformation vector selection, transformation techniques and the construction of constructs designed to express an introduced transgene. In light of the foregoing and the provision herein of Ph - hSS - X constructs, it is thus apparent that one of skill in the art will be able to introduce these constructs into plants in order to produce plants that express the desired protein (X) in their seeds.

c. Plant Types

The Ph - hSS - X constructs of the present invention may be usefully expressed in a wide range of higher plants to obtain seed specific expression of selected polypeptides. The invention is expected to be particularly applicable to monocotyledonous cereal plants including barley, wheat, rice, rye, maize, tricalate, millet, sorghum, and oat forage and turf grasses. In particular, the transformation methods described herein will enable the invention to be used with genotypes of barley including Morex, Harrington, Crystal, Stander, Moravian III, Galena, Golden Promise, Steptoe, Klages and Baroness, and commercially important wheat genotypes including Yecora Rojo, Bobwhite, Karl and Anza.

The invention may also be applied to dicotyledonous plants, including, but not limited to, soybean, cotton, beans, rape/canola, alfalfa, flax, sunflower, safflower, brassica, cotton, flax, peanut, clover; vegetables such as lettuce, tomato, cucurbits, cassava, potato, carrot, radish, pea, lentils, cabbage, cauliflower, broccoli, Brussels sprouts, peppers; and tree fruits such as citrus, apples, pears, peaches, apricots, and walnuts.

d. Vector Construction

A number of recombinant vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described including those described in Pouwels et al., (1987), Weissbach and Weissbach, (1989), and Gelvin et al., (1990). Typically, plant transformation vectors include one or more ORFs under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. The selection of suitable 5' and 3' regulatory sequences for constructs of the present invention is discussed above. Dominant selectable marker genes that allow for the ready selection of transformants include those encoding antibiotic resistance genes (*e.g.*, resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin or spectinomycin) and herbicide resistance genes (*e.g.*, phosphinothricin acetyltransferase).

e. Transformation and Regeneration Techniques

Methods for the transformation and regeneration of both monocotyledonous and

dicotyledonous plant cells are known, and the appropriate transformation technique will be determined by the practitioner. The choice of method will vary with the type of plant to be transformed; those skilled in the art will recognize the suitability of particular methods for given plant types. Suitable methods may include, but are not limited to: electroporation of plant protoplasts; liposome-mediated transformation; polyethylene glycol (PEG) mediated transformation; transformation using viruses; micro-injection of plant cells; micro-projectile bombardment of plant cells; vacuum infiltration; and *Agrobacterium tumefaciens* (AT) mediated transformation. Typical procedures for transforming and regenerating plants are described in the patent documents listed at the beginning of this section.

f. Selection of Transformed Plants

Following transformation and regeneration of plants with the transformation vector, transformed plants are usually selected using a dominant selectable marker incorporated into the transformation vector. Typically, such a marker will confer antibiotic or herbicide resistance on the seedlings of transformed plants, and selection of transformants can be accomplished by exposing the seedlings to appropriate concentrations of the antibiotic.

After transformed plants are selected and grown to maturity to allow seed set, the seeds can be harvested and assayed for the expression of the expressed "X" polypeptide.

III. Use of Seed-Expressed Polypeptides

Cells transformed as above are used to regenerate plants, and seeds from the plants are allowed to mature, typically in the field, with consequent production of heterologous protein in seeds.

Polypeptides expressed in seed using the methods described herein may be harvested from the seed at any point after expression of the protein has commenced. That is, it is not necessary for the seeds to have undergone maturation prior to harvesting. The expressed proteins may be isolated from the seeds, if desired, by conventional protein purification methods. For example, the seed can be milled, then extracted with an aqueous or organic extraction medium, followed by purification of the extracted foreign protein. Alternatively, depending on the nature of the expressed protein and the intended use, the seeds may be used directly without purification of the expressed protein.

There are differences in accumulation patterns of different polypeptides or fractions within the seed. For example, GUS expression with the GPDhGN-6-9-6 transgenic line has been found to peak at about 20 days, whereas GUS expression in the GPBhGN-4-34-7-1-2 line is higher at 10-14 days than at 20 days. Thus differential expression patterns can be monitored, and peptides extracted at times of expected peak expression.

Where the protein has been targeted to a selected intracellular body, such as a protein storage vacuole, plastid, or mitochondria, the intracellular body can first be fractionated from a seed-cell homogenate, then further fractionated to obtain the desired protein in enriched or purified form.

IV. Alternative Expression Systems

The expression systems described in the Examples below are based on the barley hordein promoter/signal sequence system. However, one of skill in the art will appreciate that the invention is not limited to this particular system. Thus, in other embodiments, other promoters and other signal sequences may be employed to express polypeptides in seed of plants, particularly cereals.

Constructs employing such sequences may be represented as:

P – X or P – SS – X, wherein X is the polypeptide to be expressed (which may be a nonplant or non-seed specific or non-plant storage protein), P is a seed maturation-specific promoter, SS is a signal sequence, such as a sequence that targets a linked polypeptide to an intracellular body, such as a protein body in which the protein is stored.

The promoter P may be a seed-specific (including, but not limited to, endosperm-specific or embryo-specific) promoter. A promoter is seed specific if its expression in a seed of a plant is at least ten-fold greater than in leaves or roots of the plant when the promoter is most actively expressed during seed maturation, and is considered endosperm-specific if its expression in the endosperm is at least five-fold greater than in other tissues of a seed when the promoter is most actively expressed during seed development. Any well-known seed-specific transcription control element or promoter can be used in addition to barley hordein promoters, including, but not limited to, promoters from any gene encoding a seed storage protein, such as well-known promoters from genes encoding: a rice glutelin, oryzin, or prolamine; wheat gliadin or glutenin; maize zein or glutelin; oat glutelin; sorghum kafirin; millet pennisetin; or rye secalin, for example.

In order to increase the levels of an expressed polypeptide, it is preferable for the protein to accumulate in a subcellular location in which the polypeptide is protected from proteolysis (that is, proteolytic degradation of the polypeptide is reduced by at least 10%, more typically by 25%, and most typically by at least 50%). As a result, it is preferred to express the polypeptide as a fusion polypeptide comprising, in the same reading frame, the coding sequence for the polypeptide and the coding sequence for a peptide (referred to interchangeably as a signal, leader, transport, or targeting sequence or peptide) that causes the fusion protein to be co-translationally or post-translationally directed to a subcellular compartment or to be secreted from the cell. Preferably, the signal peptide causes the fusion protein to be directed to a protected subcellular compartment such as the vacuole. For example, a signal peptide that causes a protein to accumulate in a vacuole may be referred to as a vacuolar targeting peptide. The signal peptide is preferably located at the 5'- or 3'-end of the fusion protein. Any well-known leader or signal peptide that causes co-translational or post-translational localization of an expressed polypeptide to such a protected subcellular compartment can be used, in addition to barley hordein signal peptides. Other such signal peptides and leaders include, but are not limited to, a signal peptides from a monocot seed-specific genes such as: a glutelin (*e.g.*, from rice, wheat, corn, oat, *etc.*), prolamine, hordein, gliadin, glutenin, zein, albumin, globulin, ADP glucosepyrophosphorylase, starch synthase, branching enzyme, Em, and lea. Another exemplary

class of signal sequences are sequences effective to promote secretion of polypeptides from aleurone cells during seed germination, including the signal sequences associated with α -amylase, protease, carboxypeptidase, endoprotease, ribonuclease, Dnase/Rnase, (1-3)- β -glucanase, (1-3)(1-4) β -glucanase, esterase, acid phosphatase, pentosamine, endoxylanase, β -xylopyranosidase, arabinofuranosidase, α -glucosidase, (1-6) α -glucanase, peroxidase and lysophospholipase.

The signal peptide may be cleaved by cellular enzymes co-translationally or post-translationally. Alternatively, if the signal peptide is not cleaved, the fusion polypeptide may be cleaved after purification of the fusion polypeptide, if desired. For this purpose, an amino-acid sequence may be introduced between the signal peptide and the heterologous protein to facilitate enzymatic or chemical cleavage to remove the signal peptide.

The following examples serve to illustrate the invention.

EXAMPLES

Example 1: Generation of Ph - hSS - X Constructs

The polymerase chain reaction was employed to produce constructs for introduction into plants. The methods used were variations of those described by Higuchi et al. (1990), Horton et al. (1990), Pont-Kingdon et al. (1994) and Lefebvre et al. (1995). The methods were employed to produce a Ph - hSS - X construct in which Ph was the barley endosperm-specific B1-hordein promoter, hSS was the barley B1-hordein signal sequence and X was the *Escherichia coli* β -glucuronidase (*uidA* ; *gus*) ORF. The construct further included the nopaline synthase (*nos*) 3' terminator. Two PCR construction methods were used: a four primer method illustrated in Fig. 1A, and a three primer method illustrated in Fig. 1B.

All PCR reactions were performed on a thermocycler (MJ Research Inc.) using recombinant *Pfu* DNA polymerase (Stratagene) in a 100- μ l reaction volume. The reaction buffer contained 20 mM Tris-HCl (pH 8.2), 10 mM KCl, 6 mM (NH₄)₂SO₄, 2 mM MgCl₂, 0.1% Triton X-100, 10 μ g/ml nuclease-free BSA and 50 μ M of each deoxyribonucleoside triphosphate. PCR conditions were 25 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, with a final extension step at 72°C for 7 min.

The recombinant PCR strategies are shown in Figure 1. The two overlapping primers were 5'-GCGGCAACAAGTAC ATTGCATTACGTCCTGTAGAAACCCCA-3' (BC primer) (Seq. I.D. No. 5) and 5'-TGGGGTTTCTACAGGACGTAATGCAAT CGTACTTGTGCGC-3' (cb primer) (Seq. I.D. No. 6); the sequence of the cb primer is the reverse complement of the BC primer. These primers contain part of the *uidA* coding sequence and part of the signal peptide coding sequence from the B1-hordein promoter. The two external primers were 5' GTAAAGCTTTAACAACCCACACA

TTG-3' (A primer) (Seq. I.D. No. 7) and 5'-CGGAATTCGATCTAGTA ACATAGAT GACA-3' (d primer) (Seq. I.D. No. 8), each with a unique restriction enzyme site (underlined), *Hind* III and *Eco*R I, respectively.

Bhorss (B1-hordein promoter containing the signal peptide sequence) and uidAnos (*uidA* coding sequence plus *nos* 3' terminator) fragments were obtained using the following PCR conditions. (I) For Bhorss, 20 ng of template (the 1.2-4/*Hind* III plasmid-containing genomic clone of B1 hordein Brandt, et al., 1985) and 40 pmol of primers A and cb were mixed in 100 μ l of 1X PCR buffer (Stratagene). (II) For uidAnos, 20 ng of template (the pDMC201.4 plasmid containing promoterless *uidA* and *nos* (McElroy et al., 1995); the *uidA* gene was derived from the pGUSN358->S plasmid modified by site-directed mutagenesis for vacuolar targeting studies (Farrell et al., 1990) and 40 pmol of primers BC and d were mixed in 100 μ l of 1X PCR buffer. After addition of 2.5 units of *Pfu* DNA polymerase, the reaction mixtures were overlaid with 50 μ l of mineral oil.

In the four-primer strategy, the two major PCR products in the first reaction were 0.49 kb for Bhorss and 2.07 kb for uidAnos. An aliquot of the first two sets of PCR reactions was diluted 50 times without gel purification and 5 μ l of diluted products were directly used as templates for the second PCR reaction. Forty pmol of external primers (A and d primers) and 2.5 units of *Pfu* DNA polymerase were added to 100 μ l of 1X PCR buffer. For the three-primer strategy, a shorter fragment of 0.49 kb Bhorss was produced by PCR in the first reaction and this first PCR product (termed megaprimer) was diluted 50 times. For the second PCR reaction, five μ l of the diluted Bhorss megaprimer (Bhorss), twenty ng of template (pDMC201.4) and 40 pmol of external primers (A and d) were mixed to a final volume of 100 μ l in 1X PCR buffer.

The second set of PCR reactions using both the modified three- and four-primer strategies produced a 2.56 kb fragment containing the Bhorss and uidAnos DNA fragments (Fig. 1C). A smaller amount of the chimeric product was produced using the 3-primer strategy relative to the 4-primer strategy. A third PCR reaction was carried out to obtain sufficient amounts of the chimeric product for microprojectile bombardment. The PCR product from the 3-primer strategy was diluted 50-fold; 5 μ l of this diluted product were added as template to 40 pmol of A/d primers in 100 μ l of 1X PCR buffer. The final chimeric products, amplified in a final volume of 2 X 100 μ l (2 reactions) from both 3-primer and 4-primer strategies were purified from a 0.7% agarose gel using QIAquick[®] gel extraction kit (Qiagen Inc.). The purified DNA fragments were eluted in 50 μ l 1X TE buffer, analyzed by restriction enzyme digestion and used in microprojectile bombardment experiments without further subcloning to test DNA construction strategies.

Example 2: Transient Assays in Barley Cells

Prior to microprojectile bombardment, spikes of barley (spring cultivar Golden Promise) containing immature embryos (15-25 days post-pollination) were sterilized in 20% (v/v) bleach (5.25% sodium hypochlorite) for 10-15 min, rinsed briefly 3 X 5 minutes with sterile water.

Endosperm was aseptically separated from each embryo manually and placed "groove-side down" on MS (Murashige and Skoog) basal medium (8) supplemented with 30 mg/l maltose, 1.0 mg/l thiamine-HCl, 0.25 mg/l myo-inositol, 1.0 g/l casein hydrolysate, 0.69 mg/l proline, and solidified with 3.5 g/l Phytigel™ (Sigma). DNA bombardment was performed using 1 µm gold particles (Analytical Scientific, Inc.) coated with 12.5 µl of the eluted DNA fragments (approximately 1-2 µg) using the following modifications of a published procedure (Lemaux et al., 1996). Gold particles and other components necessary for precipitation were reduced to half-volume. The DNA/microprojectile precipitate was resuspended in 36 µl of absolute ethanol; 15 µl of the suspension was used per bombardment in a Biolistic PDS-1000/He device (Bio-Rad) at 1100 psi. The bombarded endosperm tissue was incubated at 24 ± 1°C in the dark for 1 day and stained for GUS activity (Jefferson et al., 1987). The results (not shown) showed expression of GUS in the endosperm tissue and are consistent with the fact that the chimeric DNA construct produced by the PCR reactions described above is in frame thereby allowing production of a functional *uidA* gene product. Following the *in vivo* confirmation of functionality, the chimeric product was subcloned into a vector, further confirmed by DNA sequencing and used for stable transformation of barley in order to study protein targeting.

Example 3: Stable Expression of Ph – X Constructs in barley

Materials and Methods

Plants

The two-rowed spring cultivar of barley, Golden Promise, was grown in growth chambers as described previously (Wan and Lemaux 1994; Lemaux et al. 1996).

Nucleic Acids

Plasmid p16 (Sorensen et al. 1996) contains a pUC18 backbone with the β-glucuronidase gene (*uidA*; *gus*) controlled by 550 bp of the barley endosperm-specific B₁-hordein promoter and terminated by *Agrobacterium tumefaciens* nopaline synthase 3' polyadenylation signal, *nos*. Plasmid pD11-Hor3 (Sorensen et al. 1996) contains *uidA* controlled by 434 bp of the D-hordein promoter and a *nos* terminator. pAHC20 (Christensen and Quail 1996) contains *bar* driven by the maize ubiquitin promoter, first intron and terminated by the *nos* 3'-end. Both of these constructs include hordein promoters but do not include a hordein signal sequence. Thus, they are constructs of the type: Ph – X. pAHC25 (Christensen and Quail 1996) consists of *uidA* and *bar*, each under the control of the maize ubiquitin (*Ubi1*) promoter and first intron and terminated by *nos*.

Transformation Methods

Stable transgenic lines of barley containing B₁-hordein-*uidA* and D-hordein-*uidA* were obtained following modifications of a published protocol (Wan and Lemaux 1994; Lemaux et al. 1996). The modifications were required for transformation of commercial genotypes of barley, and the method described herein may be used to transform commercial genotypes of monocots including

barley and wheat that are recalcitrant to transformation using published methods (for example, the barley genotypes Harrington, Morex, Crystal, Stander, Moravian III, Galena, Salome, Steptoe, Klages and Baronesse and the wheat genotypes Anza, Karl, Bobwhite and Yecora Rojo).

Donor barley plants for immature embryos were grown in soil under controlled conditions in growth chambers as described (Wan and Lemaux, 1994; Lemaux *et al.*, 1996). Immature zygotic embryos were surface-sterilized, placed scutellum-side down on DBC2 medium, and incubated at $24 \pm 1^\circ\text{C}$. Regenerative tissues were maintained for 3-4 weeks, then cut into small pieces (about 3 to 5 mm), transferred to fresh DBC2 medium, and grown under dim light conditions. After an additional three weeks, green callusing sectors were broken into pieces (about 3 to 5 mm) and transferred to fresh DBC2 medium. Green regenerative tissues were maintained on DBC2 medium with subculturing at 3- to 4-week intervals.

For bombardment, green regenerative tissues (about 3 to 5 mm, four-months old) were placed in the dark at $24 \pm 1^\circ\text{C}$ for one day, then transferred to DBC2 medium containing 0.2 M mannitol and 0.2 M sorbitol. Four hours after treatment with the osmoticum, green tissues were bombarded as described by Lemaux *et al.* (1996) with gold particles (Analytical Scientific Instruments, Alameda, CA) coated with pAHC25, a mixture of pAHC20 and p16 (1:2 molar ratio), or a mixture of pAHC20 and pD11-Hor3 (1:2 molar ratio). At 16-18 hours after bombardment, the green tissues were transferred to DBC2 medium without osmoticum and grown at $24 \pm 1^\circ\text{C}$ under dim light conditions (about 10 μE , 16 h-light).

Following an initial 3- to 4-week culturing period on nonselective medium, each piece of green tissue was broken into 1 to 2 pieces (about 4 mm to 5 mm, depending on size of original tissue piece) and transferred to DBC2 medium supplemented with 5 mg/L bialaphos for *bar* selection. Green tissues were selected on DBC2 medium and 4 mm to 5 mm tissues subcultured at 3- to 4-week intervals. Bialaphos-resistant calli were regenerated on FHG (Hunter 1988) medium containing 1 mg/L 6-benzylaminopurine (BAP) and 3 mg/L bialaphos. Regenerated shoots were transferred to Magenta boxes containing rooting medium (callus-induction medium without phytohormones) containing 3 mg/L bialaphos. When shoots reached the top of the box, plantlets were transferred to soil and grown to maturity in the greenhouse.

The DBC2 medium used for transformation is based on MS medium (Murashige and Skoog, 1962) supplemented with 30 g/L maltose, 1.0 mg/L thiamine-HCl, 0.25 g/L *myo*-inositol, 1.0 g/L casein hydrolysate, 0.69 g/L proline, and solidified with 3.5 g/L Phytigel (Sigma, St. Louis, MO). The medium was further supplemented with 2.5 mg/L of the plant auxin 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/L of the plant cytokinin 6-benzylamino purine (BAP) and 5 μM copper (as copper sulfate). The presence of elevated copper and the high auxin/low cytokinin ratio were found to be required for efficient generation of the green regenerative material from genotypes of barley and wheat that are otherwise recalcitrant to transformation. However, the composition of the DBC2 medium can be varied depending on the particular genotype that is to be transformed. The key ingredients of the medium will be within the following ranges: an auxin at a concentration of about 0.1 mg/L to about 5 mg/L, a cytokinin at a concentration of 0 mg/L to

about 5 mg/L and copper at a concentration of about 0.1 μ M to about 50 μ M (and more typically about 1 to 10 μ M). More efficient transformation may be obtained when maltose is employed as a carbon source in the medium at a concentration of up to about 60 g/L, more typically about 30 g/L (either in place of or in combination with sucrose).

5 Histochemical staining for GUS was performed (Jefferson et al. 1987) using 5-bromo-4-chloro-3-indoxyl- β -D-glucuronic acid (X-gluc) (Gold Biotechnology, Inc., St. Louis, MO). Samples were incubated overnight at 37°C in GUS assay buffer.

Quantitative GUS activity measurements were performed by the method of Jefferson et al. (1987) using 4-methylumbelliferyl- β -D-glucuronide (MUG) substrate (Sigma, St. Louis, MO). From homozygous
10 lines a single immature endosperm was isolated at 10-14, 20 and 30 days after pollination or from mature endosperm, frozen in liquid nitrogen, and ground in GUS extraction buffer; each treatment had 4 replicates. After centrifugation the supernatants were used to determine GUS activity. Fluorescence of 4-methylumbelliferone (4-MU) (Sigma, St. Louis, MO) was measured on a TKO 100 dedicated mini
15 fluorometer (Hoefer Scientific Instruments, San Francisco, CA) at an excitation wavelength of 365 nm and an emission wavelength of 460 nm. Proteins were extracted as described previously (Jefferson 1987; Jefferson et al. 1987) and protein concentrations in extracts were measured according to Bradford (1976) using Bio-Rad reagent (Bio-Rad, Richmond, CA).

To determine herbicide sensitivity of T₀ plants and their progeny, a section of leaf blade at the 4- to 5-
leaf stage was painted using a cotton swab with a 0.25% solution (v/v) of BastaTM solution (starting
20 concentration, 200 g/L phophinothricin. Hoechst AG, Frankfurt, Germany) plus 0.1% Tween 20. Plants were scored 1 week after herbicide application.

Total genomic DNA from independent calli or leaf tissues was purified as described by Dellaporta (1993). To test for the presence of *uidA* in genomic DNA of putatively transformed lines, 250 ng of genomic DNA was amplified by PCR using the primer set, UIDA1 (5'-
25 AGCGGCCGCATTACGTCCTGTAGAAACC-3') (Seq. I.D. No. 9) and UID2R (5'-AGAGCTCTCATTGTTTGCTCCC TG-3') (Seq. I.D. No. 10). The presence of *bar* was tested using the primer set, BAR5F (5'-CATCGAGACAAGCACGGTCAACTTC-3') (Seq. I.D. No. 11) and BAR1R (5'-ATATCCGAGCGCC TCGTGCATGCG-3') (Seq. I.D. No. 12) (Lemaux et al. 1996). Amplifications were performed with *Taq* DNA polymerase (Promega, Madison, WI) in a 25- μ l reaction. Twenty-five μ l of the
30 PCR product with loading dye was electrophoresed on a 0.8% agarose gel with ethidium bromide and photographed using UV light. Presence of a 1.8-kb fragment with UIDA primers was consistent with an intact *uidA* fragment; an internal 0.34-kb fragment was produced with BAR primers. For DNA hybridization analysis, 10 μ g of total genomic DNA from leaf tissue of each line was digested with *Eco*RI and *Bam*HI, separated on a 1.0% agarose gel, transferred to Zeta-Probe GT membrane (Bio-Rad, Hercules, CA) and
35 hybridized with a radiolabeled *uidA*-specific probe following the manufacturer's instructions. The *uidA*-containing 1.8 kb *Xba*I-fragment from pBI221 was purified using a QIAEX gel extraction kit (QIAGEN,

Chatsworth, CA) and labeled with α -³²P-dCTP using random primers.

Results

Twenty-two independent stably transformed barley callus lines containing either B₁- or D-hordein promoter-*uidA* fusions were obtained in a first series of transformations. Thirteen lines were regenerable, seven B₁-hordein-*uidA* transformants and six D-hordein-*uidA* transformants. Genomic DNA from the callus of regenerable transformants was isolated. PCR analysis was performed using UIDA and BAR primers. PCR amplification resulted in a 1.8-kb intact *uidA* and a 0.34-kb internal *bar* fragment in T₁ progeny. From T₀ leaf tissue of the 13 lines tested, however, one (GPDhGN-22) did not produce a PCR-amplified fragment for *uidA* (Table 2). Following Southern hybridization of genomic DNA from leaf tissue of the 7 B₁-hordein-*uidA* and 6 D-hordein-*uidA* transformants, twelve of the thirteen transformed lines produced the expected 2.35-kb or 2.25-kb hordein-*uidA* fusion fragments. The remaining line (GPDhGN-22) did not produce any *uidA*-hybridizing fragments, although this line contained the appropriated-sized *bar*-hybridizing bands (data not shown).

Different tissues from stable transformants were tested for histochemical GUS activity. Strong GUS expression was seen in endosperm tissues transformed with both B₁- and D-hordein promoters, but not in embryo, ovary, stigma, anther or leaf tissues. GUS expression under control of the maize ubiquitin (*Ubi1*) promoter was observed in all tissues: no GUS expression was observed in the nontransformed control. Germinating roots and shoots from T₁ seed of either B₁- or D-hordein promoter-*uidA* fusion transformants also did not have observable histochemical GUS activity (data not shown).

Relative activities of the B₁- and D-hordein-*uidA* constructs were determined by fluorometric analysis of GUS in extracts of developing and mature seeds of homozygous lines (Table 1). The specific activities of GUS driven by the B₁-promoter-driven GUS had maximum levels expression at 10 to 20 days post-pollination. The D-hordein promoter showed a developmental pattern with peak specific activities at 20 to 30 days post-pollination.

Enzyme activity of phosphinothricin acetyltransferase (PAT, product of *bar*) and GUS in T₀ plants and their progeny was tested by painting leaves with Basta for PAT and by histochemical assay for GUS. Leaf tissue from T₀ plants of all thirteen independent lines exhibited Basta-resistance (Table 2). In T₁ progeny seven out of the thirteen lines tested for both *uidA* and *bar* showed a 3:1 segregation pattern for expression of GUS (Table 2). Of the remaining six lines, one line (GPDhGN-6) had a 43:2 segregation ratio for GUS expression; one line (GPDhGN-22) expressed PAT but did not contain *uidA*, one line (GPBhGN-13) did not contain both *uidA* and *bar*, and three lines (GPBhGN-2, GPDhGN-12 and GPDhGN-14) were sterile. T₁ endosperm from all fertile T₀ transgenic lines having positive DNA hybridization signals for *uidA* [2.35-kb and 2.25-kb fragments for the B₁-hordein-*uidA* and D-hordein-*uidA* genes, respectively], exhibited strong GUS activity (Table 2) except GPBhGN-13 and GPDhGN-14. The *bar* gene was stably transmitted to T₁ progeny of all fertile lines except GPBhGN-13; one stably expressing homozygous line (GPDhGN-16) was

obtained (Table 2). Expression of *uidA* driven by either the B₁- or D-hordein promoter was also stably inherited in T₂ progeny of all 7 independent lines tested (GPBhGN-4, -7, -12, -14, GPDhGN-6, -11 and 16) (Table 1). Expression of the *uidA* gene was stably transmitted in the one line tested at the T₅ generation (GPBhGN-4), in the two lines tested at T₄ (GPBhGN-7 and GPDhGN-16), 3 lines at T₃ (GPBhGN-12, GPDhGN-6 and -11), 1 line at T₂ (GPBhGN-14) and 1 line at T₁ (GPBhGN-3) (Table 1). Homozygous transgenic lines stably expressing *uidA* were obtained from events GPBhGN-4, -7, GPDhGN-6 and -16 (Tables 1 and 2).

Table 1. Analysis of T₀ barley plants and their progeny transformed with B₁- and D-hordein-uidA fusions

Plasmids used for bombardment	T ₀		T ₁		T ₂		T ₃		T ₄		T ₅	
	Bar	uidA (PCR)	uidA (+/-)	bar (+/-)	uidA (+/-)	bar (+/-)	uidA (+/-)	bar (+/-)	uidA (+/-)	bar (+/-)	uidA (+/-)	bar (+/-)
p16												
+pAHC20												
B2 (GPBhGN-2)*a	+	+										
B3 (GPBhGN-3)	+	+	11/3									
B4 (GPBhGN-4)	+	+	70/20	25/10	17/0	0/14						
B4-34									41/0			
B4-34-6-3									51/0			
B4-34-6-4									39/0			
B4-34-6-5									35/0			
B4-34-6-6												
B4-34-7							48/0	0/9				
B4-34-7-1									38/0	0/8		
B4-34-7-2									26/0			
B4-34-7-3									23/0			
B4-34-7-4									25/0			
B4-34-7-1-1											22/0	
B4-34-7-1-2											73/0	
B7 (GPBhGN-7)	+	+	45/18	18/6	25/0	13/1	32/0	14/4	20/0	59/0	38/0	24/0
B7-2												
B7-2-1												
B7-2-1-1												
B7-2-1-2												
B7-2-1-3												
B7-2-2-4												
B7-2-2							n.t.	6/7				

B12 (GPBhGN-12)**	+	+	21/12	15/2	9/11	5/2	4/9	1/2
B12-2								
B12-2-2								
B12-2-3								
B12-4					2/0	6/4		
B13 (GPBhGN-13)**	+	+	0/21	0/46				
B13-1					0/26	2/14	0/20	0/27
B13-1-8								
B13-1-12								
B13-2					0/17	0/15		
B14 (GPBhGN-14)**	+	+	19/12	20/9				
B14-1					10/1	11/1		
D6 (GPDhGN-6)**	+	+	43/2	8/7	40:1	2/10	18/1	51:0
D6-3							60:1	
D6-3-3								
D6-3-4								
D6-3-5								
D6-9					24:1	1/7	24/0	81:0
D6-9-1							39/1	
D6-9-6								
D6-9-7								
D11 (GPDhGN-11)**	+	+	17/10	2/1				
D11-2					1/0	9/1		
D11-2-3							2/1	

PD11-Hor3
+ pAHC20

D12 (GPDhGN-12)*a	+	+							
D14 (GPDhGN-14)*b	+	+	n.t. ^c	1/2	0/20	12/5			
D14-1							0/28		
D14-1-9							0/29		
D14-1-10							0/25		
D14-1-13							0/14		
D16 (GPDhGN-16)**	+	+	7/2	7/1	25/18	4/0	4/0	15/0	
D16-5									19/0
D16-5-2									9/0
D16-5-2-3									10/0
D16-5-2-7									5/0
D16-5-2-8									
D16-5-2-10									
D22 (GPDhGN-22)*a	+	-							

Expression of *bar* and *uidA* was tested by Basta painting and histochemical GUS assay, respectively, except for confirmation of *uidA* in T₀ plants by PCR

*Sterile

**Tetraploid

^aChromosomes were not counted

^bOutcrossed with nontransgenic plants

^cn.t.; not tested

Table 2. Specific GUS activities in developing and mature transgenic barley seeds

Days after pollination	GUS activity (pmol/min/mg protein)			
	10-14	20	30	mature
Transgenic line				
GPBhGN-4-34-7-1-2	3514 \pm 1326	2309 \pm 454	1313 \pm 340	106 \pm 37
GPDhGN-6-9-6	402 \pm 363	3812 \pm 969	2806 \pm 949	281 \pm 52
Nontransgenic control	80 \pm 31	81 \pm 23	36 \pm 13	43 \pm 9

GUS activity was determined by the fluorometric assays of protein extracts from developing and mature endosperm of homozygous lines. Values for GUS activity represent mean \pm standard deviation of four replicates for each treatment

GPBhGN-4-34-7-1-2 and GPDhGN-6-9-6 are homozygous lines transformed with B₁-hordein-*uidA* (p16) and D-hordein-*uidA* (pD11-Her3) constructs, respectively, producing T₅ and T₃ seeds, respectively

Example 4: Comparison of Seed Expression in Barley Plants Stably Transformed with Ph – hSS – X or Ph – X Constructs

To ascertain the effect of the hordein signal sequence on protein expression levels, barley plants were transformed with constructs in which the hordein B₁ promoter was operably linked to the *uidA* gene either with the hordein B₁ signal sequence (construct pdBhssGN5-6) or without the signal sequence (construct pdBhGN1-2) as illustrated in Fig. 2. Details and results of these procedures are described below.

Materials and Methods

Plasmids

Two DNA constructs containing the B₁-hordein promoter and *gus* coding region with or without the signal peptide sequence were made to test the functionality of the B₁-hordein promoter-*gus* fusions and to study its targeting:

(1) pdBhssGN5-6 (with signal sequence, Fig. 2A): the chimeric DNA construct containing the B₁-hordein promoter-signal sequence-*uidA*-*nos*, produced using the PCR methods described in Example 1, was digested with *Hind*III and *Sna*BI, and the *Hind*III/*Sna*BI fragment was ligated into *Hind*III/*Sna*BI-digested pDMC201.4 (McElroy et al., 1995) to generate the pdBhssGN5-6 plasmid. The pDMC201.4 plasmid contains promoterless *uidA* and *nos*; the *uidA* gene was derived from the pGUSN358->S plasmid modified by site-directed mutagenesis for vacuolar targeting studies (Farrell and Beachy, 1990). The PCR-amplified fragment (B₁-hordein promoter with its signal peptide sequence plus the junction region with the 5' *uidA*) of the chimeric product was confirmed by DNA sequencing.

(2) pdBhGN1-2 (without signal sequence, Fig. 2B): primers Bhor3 (5'-cgcacgcGTGCAGGTGTATGAGTCATT-3') (Seq. I.D. No. 13) and Bhor2R (5'-

ccctctagaAGTGGATTGGTGTAACT-3') (Seq. I.D. No. 14) containing *SphI* and *XbaI* sites, each with a unique restriction enzyme site (small letters), respectively, were used for amplification of 0.55-kb B₁-hordein 5' region using the p16 plasmid containing B₁-hordein promoter-*uidA-nos* (Sørensen et al., 1996) as a template. The 0.55-kb PCR-amplified fragment was digested with *SphI* and *XbaI* and ligated into *SphI/XbaI*-digested pUC19 to generate pBhor-1. pBhGN-1 was made by replacing CaMV35S promoter in p35SGN-3 (containing CaMV35S promoter-*uidA-nos*) with the *SphI/XbaI* B₁-hordein fragment from pBhor-1. The *HindIII/SnaBI* fragment from pBhGN-1 was replaced with the *HindIII/SnaBI* fragment in pDMC201.4 to generate pdBhGN1-2. Thus, the 120-bp 5' B₁-hordein flanking region was deleted in both pDbhssGN5-6 and pdBhGN1-2.

The resulting two chimeric DNA constructs were introduced using microprojectile bombardment into immature barley endosperm tissues for stable gene expression assays. Stable transformation methods, GUS staining and quantitation, and Basta sensitivity were determined as described in Example 3.

Genomic DNA isolation, polymerase chain reaction (PCR) and DNA blot hybridization

Total genomic DNA from independent calli or leaf tissues was purified as described (Dellaporta, 1993). To test for the presence of *uidA* in genomic DNA of putatively transformed lines, 250 ng of genomic DNA was amplified by PCR using the primer set, UIDA1 and UID2R. The primer set, Bhor8 (5'-GAAGAGATGAAGCCTGGCTAC-3') (Seq. I.D. No. 15) and GUS5516 (5'-CGATCCAGACTGAATGCC CACAGG-3') (Seq. I.D. No. 16) was used to distinguish between the B₁-hordein promoter with and without the signal sequence. A 229-bp hybrid product is expected from the construct containing the B₁-hordein promoter with the signal sequence while a 178-bp PCR product is expected from the construct containing the B₁-hordein promoter without the signal sequence. The presence of *bar* was tested using the primer set, BAR5F and BAR1R (Lemaux et al. 1996). Amplifications were performed with *Taq* DNA polymerase (Promega, Madison, WI) in a 25- μ l reaction. Twenty-five μ l of the PCR product with loading dye was electrophoresed on a 0.8% agarose gel with ethidium bromide and photographed using UV light. Presence of a 1.8-kb fragment with UIDA primers was consistent with an intact *uidA* fragment; an internal 0.34-kb fragment was produced with BAR primers. For DNA hybridization analysis, 10 μ g of total genomic DNA from leaf tissue of each line was digested with *HindIII* and *SacI*, separated on a 1.0% agarose gel, transferred to Zeta-Probe GT membrane (Bio-Rad, Hercules, CA) and hybridized with a radiolabeled *uidA*-specific probe following manufacturer's instructions. The *uidA*-containing 1.8 kb *XbaI*-fragment from pBI221 was purified using a QIAEX gel extraction kit (QIAGEN, Chatsworth, CA) and labeled with α -³²P-dCTP using random primers.

Immunogold labeling assay

Transgenic and nontransgenic immature endosperm were harvested about 20 days after pollination using a high pressure freezing method (McDonald, 1998). The tissues were embedded in

white resin and immunocytochemistry was performed as previously described (Phillip et al., 1998)

Results

PCR and DNA blot hybridization analysis of transgenic plants

To test the functionality of the N-terminal signal peptide sequence of the B₁-hordein promoter and to study targeting mechanisms, we obtained 10 independent stably transformed barley lines containing either pdBhssGN5-6 or pdBhGN1-2. Of these, three lines from each DNA construct were co-expressed lines with *uidA* and *bar* genes. Genomic DNA from these transformants was isolated. PCR analysis was performed using UIDA and BAR primers. PCR amplification resulted in 1.8-kb intact *uidA* and 0.34-kb internal *bar* fragments from all six lines (Table 3). A 51 bp size difference between the fragments amplified from the B₁-hordein-*uidA* transformants with and without the signal sequence was observed and is accounted for by the presence of the signal sequence in lines GPdBhGN-1, -2 and -16.

Hordein-uidA expression in transgenic plants

T₁ seeds were tested for histochemical GUS activity. Strong GUS expression was seen in endosperm tissues transformed with both B₁-hordein promoters with and without the signal sequence, but not in the embryo. GUS expression from the two B₁-hordein-*uidA* constructs was very evident in developing endosperm, especially in peripheral cells of endosperm tissue and endosperm tissue near the scutellar side. The B₁-hordein promoter with the signal sequence had even stronger expression in endosperm than did that without the signal sequence. No GUS expression was observed in the nontransformed control tissues.

Relative activities of the B₁-hordein-*uidA* constructs were determined by fluorometric analysis of GUS in extracts of developing and mature seeds of homozygous lines. The specific activities of GUS driven by the B₁-hordein promoter with the signal sequence were higher in both developing and mature seeds than without the signal sequence (Figs. 6 and 7). In particular, developing endosperm tissues transformed with the B₁-hordein promoter with the signal sequence had more than 30 times GUS activity, compared with the B₁-hordein promoter without the signal sequence.

Analysis of T₀ to T₂ progeny

Enzyme activity of phosphinothricin acetyltransferase (PAT, product of *bar*) and GUS in T₀ plants and their progeny was tested by painting leaves with Basta for PAT and by histochemical assay for GUS. Leaf tissue from T₀ plants of all six independent lines exhibited Basta resistance (Table 1). In T₁ progeny all six lines tested for *uidA* showed a 3:1 segregation pattern for expression of GUS. The *bar* gene was also stably transmitted to T₁ progeny of five lines except GPdBhssGN-10; the GPdBhssGN-10 line did not express PAT. Homozygous transgenic lines stably expressing *uidA* were obtained from 4 events GPdBhGN-1, GPdBhssGN-7, -10, and -23.

Electron Microscopy and immunogold labeling

The immunosignal seen at the electron microscopic level was specific for protein bodies in GUS-expressing immature endosperm of line that was transformed with the B₁-hordein-*uidA* DNA construct containing the 19 amino acid N-terminal signal peptide sequence (Fig. 7).

Discussion

The foregoing research tested the functionality of the barley B₁-hordein promoters with and without the 19 amino acid N-terminal signal peptide sequence using both transient and stable expression assays in barley. Consistent with earlier studies (Müller and Knudsen 1993; Sørensen et al. 1996), transient expression of GUS under the control of the B₁- hordein promoter-*uidA* fusion without the signal sequence was observed in developing endosperm, but not in embryos. The B₁-hordein promoter with the signal sequence exhibited the same expression pattern, but with stronger expression. PCR analysis confirmed the presence of *uidA* and *bar* in genomic DNA from T₀ plants of 6 different lines stably transformed with B₁-hordein promoters with and without the signal sequence.

Stably transformed, developing and mature barley seeds were characterized in terms of tissue-specificity and timing of hordein promoter-driven GUS expression. GUS driven by both B₁-hordein promoters with and without the signal sequence was expressed exclusively in endosperm tissue, not in other tissues. In addition, the existence of the signal sequence with the B₁-hordein promoter dramatically enhanced GUS expression in developing transgenic endosperm. This is consistent with the results of Fiedler and Conrad (1995) that an antigen-binding single chain Fv (scFv) protein was only detectable in seeds of tobacco plants transformed with constructs containing a signal peptide sequence. After storage of mature transgenic tobacco seeds for one year at room temperature, there was no loss of scFv protein or its antigen-binding activity whereas in plants transformed with a construct without a signal peptide no detectable accumulation of scFv in ripe or developing seeds occurred. They speculated that the lack of GUS protein expression in cytosol is a result of translational or post-translational regulation by cytosolic proteases, which degrade the scFv protein if it does not enter the secretory pathway. In contrast, the levels of GUS mRNAs in developing seeds and GUS activity in mature seeds were consistently higher in transformed tobacco lines that contained the 32 amino acid N-terminal amino acid sequence of soybean embryo-specific lectin gene as compared with those that lacked this sequence (Phillip et al., unpublished observation). GUS expression from the two B₁-hordein-*uidA* constructs was very evident especially in peripheral cells of developing endosperm tissue and endosperm tissue near the scutellar side in transformed barley.

Transgenic plants with a single site of transgene integration would be expected to give a segregation ratio for the transgene (and its expression) of 3:1. All six lines gave such a ratio for GUS expression. Homozygous, stably expressing GUS transgenic lines were obtained from 4 lines.

Expression of the maize ubiquitin promoter-driven PAT was also stably inherited in T₁ progeny of five transgenic lines; however, one line (GPdBhssGN-10) did not show PAT expression in T₂ progeny.

5 The results presented here show that the D- or B₁-hordein promoter can be used to develop a system for limiting foreign gene expression exclusively to the endosperm of barley seed. In addition, the results show that the use of plants transformed with a seed-specific promoter with the signal sequence dramatically enhanced the transgene expression. This permits the creation of new crop varieties for nutraceutical and pharmaceutical purposes using vacuolar targeting strategies.

10 Example 5: Embryo Specific Expression

This example uses the maize *Glb1* promoter, with or without deletion of a 5' flanking sequence, fused to the bacterial reporter gene, β -glucuronidase gene (*uidA*; *gus*), to demonstrate the functionality of the maize *Glb1* promoter in transgenic barley, for embryo specific expression.

15 A two-rowed spring cultivar of barley, Golden Promise, was grown in growth chambers as described previously (Wan and Lemaux 1994; Lemaux et al. 1996).

Plasmids

ppGlb1GUS (Liu and Kriz, 1996), a plasmid containing *uidA* reporter gene under the control of the maize embryo-specific globulin (*Glb1*) promoter and terminated by *nos*, was obtained from DEKALB Plant Genetics, Mystic, CT. The ppGlb1GUS was digested with *EcoRI* to remove a 1.04-kb 5' globulin flanking region. The 2.58-kb *EcoRI* fragment containing the 0.36-kb globulin promoter, *uidA* coding sequence and *nos* 3' terminator was ligated into *EcoRI*-digested pUC19 to generate pdGlbGUS-6. Thus, the 1.04-kb 5' globulin flanking region was deleted in pdGlbGUS-6. The above two chimeric DNA constructs were introduced using microprojectile bombardment into immature barley endosperm tissues for both transient and stable gene expression assays.

25 *Transient gene expression of the uidA genes driven by globulin-promoters*

Spikes about 20 to 25 days after pollination were surface-sterilized for 10 to 15 min in 20% (v/v) bleach (5.25% sodium hypochlorite), followed by 3 washes with sterile water. Immature embryos and endosperm tissues were aseptically separated and placed scutellum-side up on MS medium (Murashige and Skoog 1962) supplemented with 3.5 g/L of Phytagel (Sigma, St. Louis, MO) with or without osmoticum treatment (Cho et al., 1998b). The tissues were bombarded using a Biolistic PDS-1000 He gun (Bio-Rad, Hercules, CA) at 1100 psi with 1.0 μ m gold particles coated with either ppGlb1GUS or pdGlbGUS-6 according to the protocol of Lemaux et al. (1996). Osmotic treatment includes 0.2 M mannitol and 0.2 M sorbitol to give a final concentration of 0.4 M with 4-h pre-treatment and 1- or 2-d post-treatment.

Stable barley transformations

Stable transgenic GP lines were obtained via microparticle bombardment essentially as described in Wan and Lemaux 1994; Lemaux et al. 1996; and Cho et al., 1998a. Gold particles (1.0 μm) were coated with 25 μg of 1:1 molar ratio of pAHC20 and ppGlb1GUS or pdGlbGUS-6, and used in bombardment experiments as described above. Plasmid pAHC20 (Christensen and Quail, 1996) contains the herbicide resistance gene, *bar*, from *Streptomyces hygroscopicus* under the control of the maize ubiquitin *Ubi1* promoter and first intron and *nos* 3' terminator. Bialaphos-resistant calli were regenerated on FHG (Hunter 1988) medium containing 1 mg/L 6-benzylaminopurine (BAP) and 3 mg/L bialaphos. Regenerated shoots were transferred to Magenta boxes containing rooting medium (callus-induction medium without phytohormones) containing 3 mg/L bialaphos. When shoots reached the top of the box, plantlets were transferred to soil and grown to maturity in the greenhouse.

Histochemical and quantitative assays of GUS activity

Histochemical staining for GUS was performed using 5-bromo-4-chloro-3-indoxyl- β -D-glucuronic acid (X-gluc) (Gold Biotechnology, Inc., St. Louis, MO). Samples were incubated overnight at 37°C in GUS assay buffer.

Herbicide application

To determine herbicide sensitivity of T₀ plants and their progeny, a section of leaf blade at the 4- to 5-leaf stage was painted using a cotton swab with a 0.25% solution (v/v) of BastaTM solution (starting concentration, 200 g/L phosphinothricin, Hoechst AG, Frankfurt, Germany) plus 0.1% Tween 20. Plants were scored 1 week after herbicide application.

Genomic DNA isolation, polymerase chain reaction (PCR) and DNA blot hybridization

Total genomic DNA from independent calli or leaf tissues was purified as described (Dellaporta, 1993). To test for the presence of *uidA* in genomic DNA of putatively transformed lines, 250 ng of genomic DNA was amplified by PCR using the primer set, UIDA1 (5'-agcggccgcaTTACGTCCTGTAGAAACC-3') and UID2R (5'-agagctcTCATTGTTTGCCTCCC TG-3'); each with a restriction enzyme site (small letters) for subcloning of another DNA construct containing the *uidA* gene (Cho et al. 1998a;b). The presence of *bar* was tested using the primer set, BAR5F (5'-CATCGAGACAAGCACGGTCAACTTC-3') and BAR1R (5'-ATATCCGAGCGCC TCGTGCATGCG-3') (Lemaux et al. 1996). Amplifications were performed with *Taq* DNA polymerase (Promega, Madison, WI) in a 25- μl reaction (Cho et al. 1998a;b). Twenty-five μl of the PCR product with loading dye was electrophoresed on a 0.8% agarose gel with ethidium bromide and photographed using UV light. Presence of a 1.8-kb fragment with UIDA primers was consistent

with an intact *uidA* fragment; an internal 0.34-kb fragment was produced with BAR primers.

Results

Transient gene expression of globulin-uidA genes

To establish initially the functionality of the maize embryo-specific globulin promoter, plasmids ppGlb1GUS and pdGlbGUS-6 were used in transient assays involving microprojectile bombardment into immature barley endosperm and embryos, respectively. Two controls were included, one negative control bombarded with 1xTE buffer and one positive control bombarded with pAHC25 containing *uidA* under control of the constitutive maize ubiquitin promoter. GUS driven by the maize embryo-specific globulin promoters was weakly expressed in embryo tissue, but not in endosperm tissue at all. GUS Expression driven by the two globulin promoter *uidA* fusions was stronger in barley immature embryos with osmotic treatment than without osmotic treatment. The degree of *uidA* gene expression in embryos driven by the undeleted globulin promoter (1.4-kb, ppGlb1GUS) appeared slightly weaker than that driven by the deleted globulin promoter (0.36-kb, pdGlbGUS-6). ABA treatment enhanced GUS expression in immature embryos without osmotic treatment, but not with osmotic treatment. The negative control did not exhibit any GUS expression.

Embryo specific expression in transgenic plants

To further test the maize *Glb1-uidA* constructs, ten independent stably transformed barley lines were obtained; 5 transformed with the undeleted globulin promoter and another 5 with deleted globulin promoter. Genomic DNA from regenerable transformants was isolated and PCR analysis were performed. Results of PCR amplification of *uidA* and *bar* genes from genomic DNA extracted from callus tissues showed that transgenic barley lines resulted in the generation of both the 1.8-kb intact *uidA* and the 0.34-kb internal *bar* fragments.

Different tissues from the stable transformants were tested for histochemical GUS activity. Weak *uidA* gene expression was exhibited exclusively in embryo tissues transformed with maize *Glb1* promoters, but not in the endosperm tissue. On the other hand, GUS expression under control of the maize ubiquitin (*Ubi1*) promoter was observed in all tissues; no GUS expression was detected in the negative control.

This invention has been detailed both by example and by description. It should be apparent that one having ordinary skill in the relevant art would be able to surmise equivalents to the invention as described in the claims which follow but which would be within the spirit of the foregoing description. Those equivalents are to be included within the scope of this invention.

Analysis of T₀ barley plants and their progeny transformed with B₁-hordein-*uidA* fusions with and without signal sequence.

Plasmids used for bombardment	Transgenic Barley Line	DNA PCR <i>uidA</i>	PCR (T ₀) <i>bar</i>	Basta resistant (T ₀)	GUS activity in T ₁ seeds (+/-)	Basta resistance in T ₁ plants (+/-)	Ploidy (T ₀)	Comments
pdBhGN1-2 + pAHC20	GPdBhGN-1	+	+	+	+	(47:25)	diploid	homozygous
	GPdBhGN-2	+	+	+	+	(11:3)	tetraploid	
	GPdBhGN-16	+	+	+	+	(61:31)	tetraploid	
pdBhssGN5-6 + pAHC20	GPdBhssGN-7	+	+	+	+	(146:40)	diploid	homozygous
	GPdBhssGN-10	+	+	+	+	(136:41)	diploid	homozygous
	GPdBhssGN-23	+	+	+	+	(148:42)	diploid	homozygous

TABLE 3

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We claim:

1. A recombinant nucleic acid molecule having a structure

$$P - X \text{ or } P - SS - X,$$

wherein X is a nucleic acid molecule encoding a polypeptide, P is a seed maturation-specific promoter, and SS is a signal sequence that targets a linked polypeptide to an intracellular body.
2. The recombinant nucleic acid molecule of claim 1, wherein the polypeptide is other than a seed storage protein.
3. The recombinant nucleic acid molecule of claim 1, wherein the polypeptide is other than a seed specific protein.
4. The recombinant nucleic acid molecule of claim 1, wherein the signal sequence targets a linked polypeptide to a protein storage body.
5. A transgenic plant comprising a nucleic acid molecule according to claim 1.
6. Seed of a transgenic plant according to claim 5.
7. The plant of claim 5 wherein the plant is a stably transformed monocotylenous plant.
8. The plant of claim 7, wherein the monocotylenous plant is a cereal selected from the group of rice, barley, maize, wheat, oat, rye, sorghum, millet, or tricalate.
9. The plant of claim 8 wherein the plant is a barley, corn or wheat plant.
10. Seed of a transgenic wheat or barley plant according to claim 9.
11. Seed of a transgenic plant of claim 8.
12. A transgenic plant according to claim 5 wherein, in the nucleic acid molecule, P is a barley hordein promoter, and SS is a barley hordein signal peptide.
13. A transgenic plant according to claim 12 wherein the barley hordein promoter is selected from the group consisting of barley B1 and D hordein promoters, and the barley hordein signal peptide is selected from the group consisting of barley B1 and D hordein signal peptides.
14. The seed of claim 10, wherein the polypeptide is purified from the seed at a preselected time during seed maturation at which the polypeptide is expressed at a desired concentration.
15. The transgenic plant of claim 5, wherein expression of the polypeptide increases the concentration of a plant metabolite.
16. A recombinant nucleic acid molecule having a structure

$$Ph - hSS - X,$$

wherein Ph is a hordein promoter, hSS is a hordein signal sequence, and X is a nucleic acid molecule encoding a polypeptide, and where Ph, hSS and X are operably linked.
17. A transgenic plant comprising a nucleic acid molecule according to claim 16.
18. Seed of a transgenic plant according to claim 17.

19. The plant of claim 16 wherein the plant is a stably transformed monocotyledenous plant.
20. The plant of claim 19, wherein the monocotyledenous plant is a cereal selected from the group of rice, barley, maize, wheat, oat, rye, sorghum, millet, or tricalate.
- 5 21. The plant of claim 20 wherein the plant is a barley, corn or wheat plant.
22. Seed of a transgenic wheat or barley plant according to claim 21.
23. A transgenic barley plant according to claim 20 wherein the barley plant is a genotype selected from the group consisting of: Harrington, Morex, Crystal, Golden Promise, Stander, Moravian III, Galena, Salome, Steptoe, Klages and Baronesse.
- 10 24. Seed of a transgenic barley plant according to claim 22.
25. A transgenic wheat plant according to claim 22 wherein the wheat plant is a genotype selected from the group consisting of: Anza, Karl, Bobwhite and Yecora Rojo.
26. Seed of a transgenic wheat plant according to claim 25.
27. A method of expressing a polypeptide in seed of a monocotyledenous plant,
- 15 comprising:
- (a) providing a monocotyledenous plant that is stably transformed with a nucleic acid molecule according to claim 1; and
- (b) growing the seeds under conditions effective to produce seed and express the polypeptide in the seed.
- 20 28. A method of expressing a polypeptide in seed of a monocotyledenous plant, comprising:
- (a) providing a monocotyledenous plant that is stably transformed with a nucleic acid molecule according to claim 15; and
- (b) growing the seeds under conditions effective to produce seed and express the polypeptide in the seed.
- 25 29. The method of claim 28 wherein the plant is a barley, corn or wheat plant.
30. A method of producing a stably transformed monocotyledenous plant expressing a selected polypeptide in seeds of the plant, comprising:
- (a) placing an immature zygotic embryo of the plant on plant growth medium comprising maltose as a sugar source, an auxin at a concentration of about 0.1 mg/L to about 5 mg/L, a cytokinin at a concentration of 0 mg/L to about 5 mg/L and copper at a concentration of about 0.1 μ M to about 50 μ M, and incubating in dim light conditions so as to form green regenerative tissue;
- 35 (b) introducing a nucleic acid molecule into the tissue to produce transformed tissue, wherein the nucleic acid molecule has a structure
- $$\text{Ph} - \text{hSS} - \text{X},$$
- wherein Ph is a hordein promoter, hSS is a hordein signal sequence, and X is a nucleic acid

molecule encoding the selected polypeptide, and where Ph, hSS and X are operably linked;

(c) incubating the transformed tissue on the plant growth medium such that green structures are observed on the transformed material;

(d) regenerating at least one transformed plant from the green structures; and

5 (f) growing the transformed plant to produce seed.

31. The method of claim 30, wherein the monocotyledonous plant is a cereal selected from the group of rice, barley, maize, wheat, oat, rye, sorghum, millet, or tricalate.

32. The method of claim 31 wherein the plant is a barley, corn or wheat plant.

33. The method of claim 32 wherein the barley plant is a genotype selected from the group consisting of: Harrington, Morex, Crystal, Golden Promise, Stander, Moravian III, Galena, Salome, Steptoe, Klages and Baronesse.

34. The method of claim 32 wherein the wheat plant is a genotype selected from the group consisting of: Anza, Karl, Bobwhite and Yecora Rojo.

35. A method of producing a stably transformed monocotyledonous plant expressing a selected polypeptide in seeds of the plant, comprising:

15 (a) placing an immature zygotic embryo of the plant on plant growth medium comprising maltose as a sugar source, an auxin at a concentration of about 0.1 mg/L to about 5 mg/L, a cytokinin at a concentration of 0 mg/L to about 5 mg/L and copper at a concentration of about 0.1 μ M to about 50 μ M, and incubating in dim light conditions so as to form green regenerative tissue;

(b) introducing a nucleic acid molecule into the tissue by to produce transformed tissue, wherein the nucleic acid molecule has a structure

Ph - X,

25 wherein Ph is a hordein promoter and X is a nucleic acid molecule encoding the selected polypeptide, and where Ph and X are operably linked;

(c) incubating the transformed tissue on the plant growth medium such that green structures are observed on the transformed material;

(d) regenerating at least one transformed plant from the green structures; and

(f) growing the transformed plant to produce seed.

30 36. The method of claim 35 wherein the plant is a barley, corn or wheat plant.

37. The method of claim 36 wherein the barley plant is a genotype selected from the group consisting of: Harrington, Morex, Crystal, Stander, Moravian III, Galena, Salome, Steptoe, Klages and Baronesse.

38. The method of claim 36 wherein the wheat plant is a genotype selected from the group consisting of: Anza, Karl, Bobwhite and Yecora Rojo.

39. A method of producing a stably transformed monocotyledonous plant expressing a selected polypeptide in seeds of the plant, comprising:

(a) placing an immature zygotic embryo of the plant on plant growth medium comprising maltose as a sugar source, an auxin at a concentration of about 0.1 mg/L to about 5 mg/L, a cytokinin at a concentration of 0 mg/L to about 5 mg/L and copper at a concentration of about 0.1 μ M to about 50 μ M, and incubating in dim light conditions so as to form green
 5 regenerative tissue;

(b) introducing a nucleic acid molecule into the tissue by to produce transformed tissue, wherein the nucleic acid molecule has a structure



wherein X is a nucleic acid molecule encoding a polypeptide, P is a seed maturation-specific promoter, and SS is a signal sequence that targets a linked polypeptide to an intracellular body;
 10

(c) incubating the transformed tissue on the plant growth medium such that green structures are observed on the transformed material;

(d) regenerating at least one transformed plant from the green structures; and

(f) growing the transformed plant to produce seed.

15 40. The method of claim 39 wherein the plant is a barley or a wheat plant.

41. The method of claim 39 wherein, in the nucleic acid molecule, P is a barley hordein promoter, and SS is a barley hordein signal peptide.

20 42. The method of claim 41 wherein the barley hordein promoter is selected from the group consisting of barley B1 and D hordein promoters, and the barley hordein signal peptide is selected from the group consisting of barley B1 and D hordein signal peptides.

43. The recombinant nucleic acid of claim 1, wherein the promoter is selected from the group of seed maturation specific promoters comprising rice glutelins, rice oryzins, rice prolamines, barley hordeins, wheat gliadins, wheat glutelins, maize zeins, maize glutelins, oat glutelins, sorghum kasirins, millet pennisetins, and rye secalins.

25 44. The recombinant nucleic acid of claim 1 wherein the signal sequence is selected from the group of a signal peptide from a monocot seed-specific gene, including a glutelin, prolamine, hordein, gliadin, glutenin, zein, albumin, globulin, ADP glucosepyrophosphorylase, starch synthase, branching enzyme, Em, and lea.

30 45. The recombinant nucleic acid of claim 1, wherein the signal sequence is selected from signal sequences that promote secretion of polypeptides from aleurone cells during seed germination.

35 46. The recombinant nucleic acid of claim 45, wherein the signal sequence is selected from a signal sequences associated with α -amylase, protease, carboxypeptidase, endoprotease, ribonuclease, Dnase/Rnase, (1-3)- β -glucanase, (1-3)(1-4) β -glucanase, esterase, acid phosphatase, pentosamine, endoxylanase, β -xylopyranosidase, arabinofuranosidase, α -glucosidase, (1-6) α -glucanase, peroxidase or lysophospholipase.

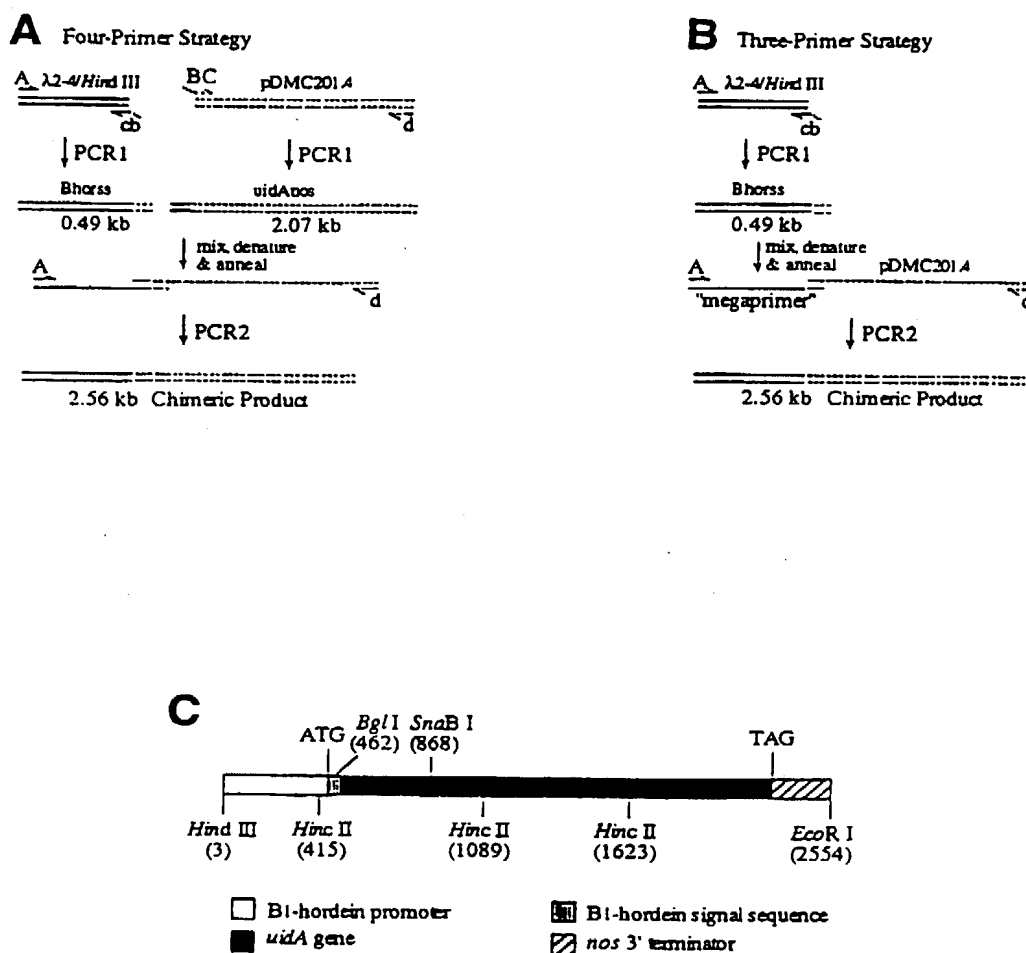


FIG. 1
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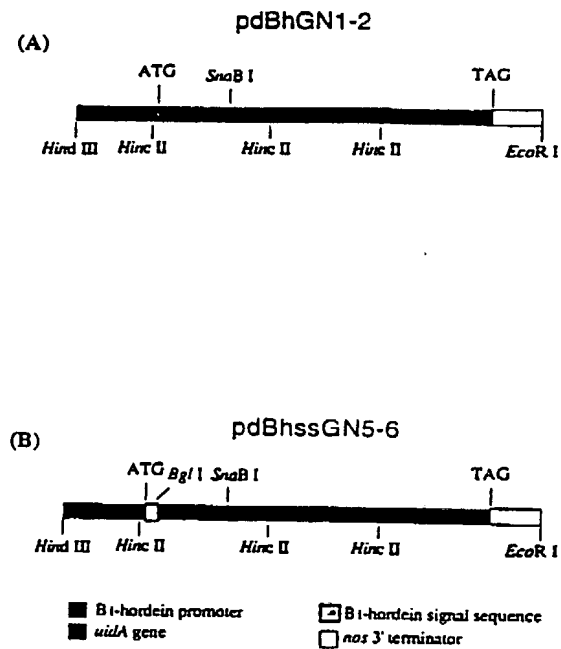


FIG. 2
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AAGCTTTAACAAACCCACACATTGATTGCAACTTAGTCCTACACAAAGTTTCCATT
CTTGTTTCAGGCTAACAAACCTATACAGGTTCCAAATCATGCAAAAGTGATGC
TAGGTTGATAATGTGTGACATGTAAGTGATAAGTGAGTGCATGCATACCAA
CCTCGGATTCTATACITTTGTGTATGATCATATGCACAACTAAAGGCAACTTT
GATTATCAATTGAAAAGTACCGCTTGTAGCTTGTGCAACCTAACACAATGTCCA
AAATCCATTGTGCAAAAGCATCCAAACACAATTGTTAAGCTGTTCAACAAC
AAAGAGAGATGAAGCTGGCTACTATAAATAGGCAGGTAGTATAGAGATCTA
CACAAAGCACAAAGCATCAAAACCAAGAACACTAGTTAACACCAATCCACTATGA
AGACCTTCCTCATCTTTGCACTCCTCGCCATTGCGGCAACAAGTACGATTGCA

FIG. 3

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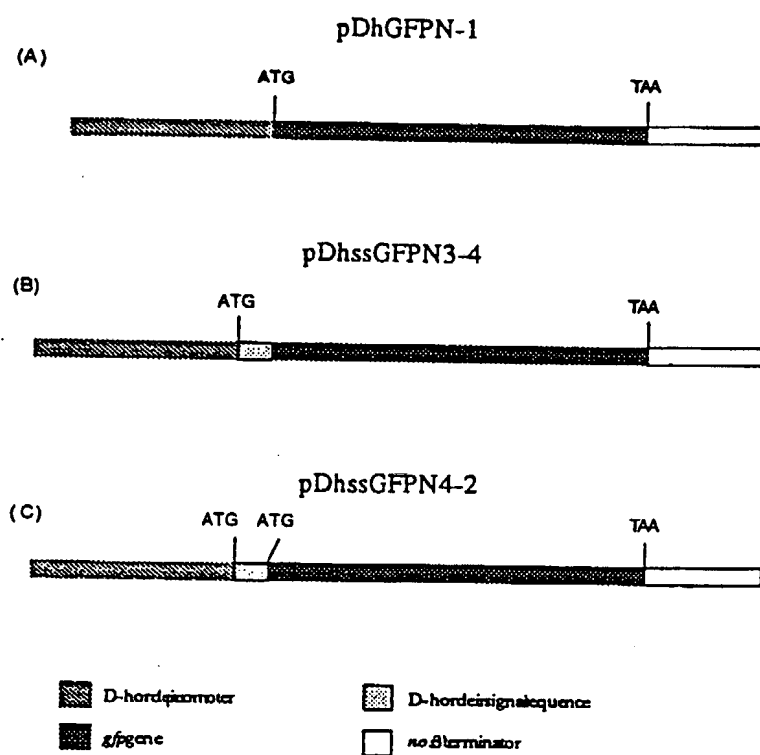


FIG. 4
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CTTCGAGTCCCCCGGATTTGCCAGCAATGGCTAACAGACACATATTCTGCC
AAACCCCGAGAACATAATCACTTCGTAGATGAAGAGAACAGACCAAGAT
ACAAACGTCCACGCTTCAGCAACAGTACCCAGAACTAGGATTAAGCCGAT
TACCGGGCTTAGCAGACCGTCCAAATACTGTTTGGCAAGCTCCAAATCC
TCCTTGCTTATCCAAATTTCTTTTGTGTTGGCAAACTGCACTTGTCACACCGATT
TTGTTCTTCCCGTGTTCCTTCTTAGGCTAACTAACACAGCCGTGCACATAGCC
ATGGTCCGGAATCTTCACCTCGTCCCTATAAAGCCAGCCAACTCTCCACAAT
CTCATCATCACCGAGAACACCGAGAACCAAACTAGAGATCAATTTCATTG
ACAGTCCACCGAGATGGCTAAGCGGTGCTCTCTTTGTGGCGGTAAATCGTC
GCCCTCGTGGCTCTCACCCCGCT

FIG. 5

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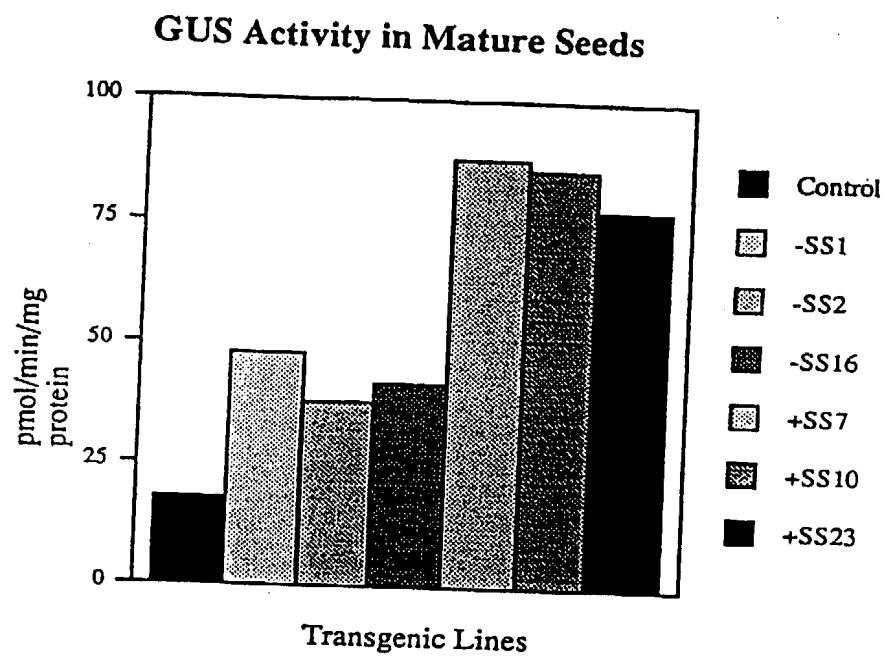


FIG. 6
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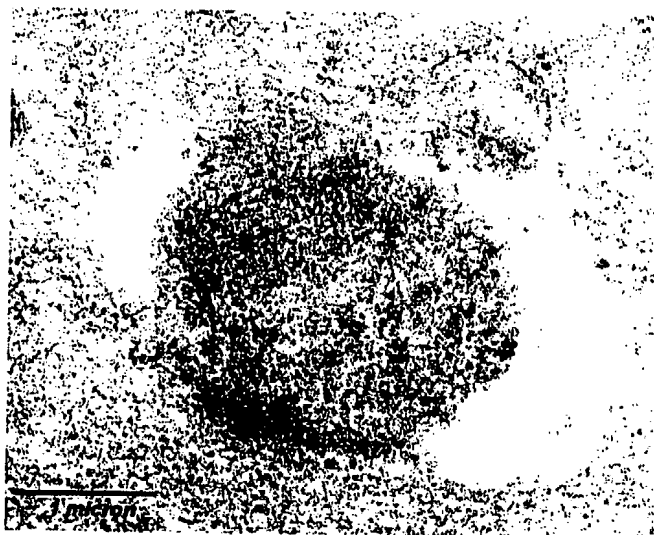


FIG. 7
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US98/20691 (22) International Filing Date: 30 September 1998 (30.09.98) (30) Priority Data: 60/060,510 30 September 1997 (30.09.97) US (71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; University of California, Berkeley, Office of Technology Licensing, Suite 510, 2150 Shattuck Avenue, Berkeley, CA 94720-1620 (US). (72) Inventors: LEMAUX, Peggy, G.; 253 Corliss Drive, Moraga, CA 94556 (US). CHO, Myeong-Je; 3419 Solomon Avenue, Alameda, CA 94502 (US). BUCHANAN, Robert, B.; 19 Tamalpais Road, Berkeley, CA 94708 (US). (74) Agent: NOONAN, William, D.; Klarquist, Sparkman, Campbell, Leigh & Winston, LLP, One World Trade Center, Suite 1600, 121 S.W. Salmon Street, Portland, OR 97204 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 20 May 1999 (20.05.99)
(54) Title: PRODUCTION OF PROTEINS IN PLANT SEEDS (57) Abstract Methods for producing proteins in plant seeds are disclosed. Expression of the protein is driven by a seed-specific promoter and the protein is preferably expressed as a fusion polypeptide that includes a signal peptide that causes the protein to accumulate in a subcellular compartment to protect the protein. Also disclosed are improved methods for transforming plants, including cereals such as barley, corn or wheat.		

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/20691

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHO M ET AL: "Expression of hordein promoter-uidA fusions in transgenic barley plants; endosperm-specific B- and D-hordein promoter gene expression in transgenic plant, for potential crop improvement" BIOTECHABS, vol. 3, no. 32, 1996, page 103a XP002054427 see abstract	1, 3, 5-11, 27, 43
X	BLOM SORENSEN M ET AL: "HORDEIN PROMOTER METHYLATION AND TRANSCRIPTIONAL ACTIVITY IN WILD-TYPE AND MUTANT BARLEY ENDOSPERM" MOLECULAR AND GENERAL GENETICS, vol. 250, no. 6, 10 April 1996, pages 750-760, XP002054426 cited in the application	1-3, 43
A	see the whole document	4-42
	-/-	

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Date of the actual completion of the international search

17 March 1999

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INTERNATIONAL SEARCH REPORT

Int. onal Application No

PCT/US 98/20691

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>TORRENT M ET AL: "LYSINE-RICH MODIFIED GAMMA-ZEINS ACCUMULATE IN PROTEIN BODIES OF TRANSIENTLY TRANSFORMED MAIZE ENDOSPERMS"</p> <p>PLANT MOLECULAR BIOLOGY, vol. 34, no. 1, 1 May 1997, pages 139-149, XP002034740 see esp. p.145 r.col - p.146 l.col; p.148 l.par.</p> <p style="text-align: center;">---</p>	<p>1,4-9, 11,15, 27-29, 43,44</p>
X	<p>ZHANG Z. ET AL.: "The bean seed storage protein beta-phaseolin is synthesized, processed, and accumulated in the vacuolar type-II protein bodies of transgenic rice endosperm"</p> <p>PLANT PHYSIOLOGY, vol. 109, no. 3, November 1995, pages 777-786, XP002096844 cited in the application see esp. p.784 r.col. 2.par.</p> <p style="text-align: center;">---</p>	<p>1,4-8, 11,15,43</p>
X	<p>WO 97 29200 A (INST PFLANZENGENETIK UND KULTU ;CONRAD UDO (DE); FIEDLER ULRIKE (D) 14 August 1997 see esp. examples 1,2,4</p> <p style="text-align: center;">---</p>	<p>1-6</p>
X	<p>WO 95 15392 A (DU PONT ;FALCO SAVERIO CARL (US); KEELER SHARON JO (US); RICE JANE) 8 June 1995 see esp. examples 12,13</p> <p style="text-align: center;">---</p>	<p>1-9,11, 15, 27-29,43</p>
X	<p>WO 97 25419 A (US GOVERNMENT) 17 July 1997</p> <p>see the whole document</p> <p style="text-align: center;">---</p>	<p>1,4-11, 15, 27-29, 43,44</p>
X	<p>WO 94 20628 A (DU PONT) 15 September 1994</p> <p>see the whole document</p> <p style="text-align: center;">---</p>	<p>1,4-6, 15,44</p>
X	<p>WO 91 13993 A (UPJOHN CO) 19 September 1991 see esp. examples 6,10</p> <p style="text-align: center;">---</p>	<p>1-4,27, 43,44</p>
X	<p>WO 90 01551 A (ROGERS JOHN C) 22 February 1990</p> <p>see the whole document</p> <p style="text-align: center;">---</p>	<p>1-11,15, 27-29, 45,46</p>
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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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